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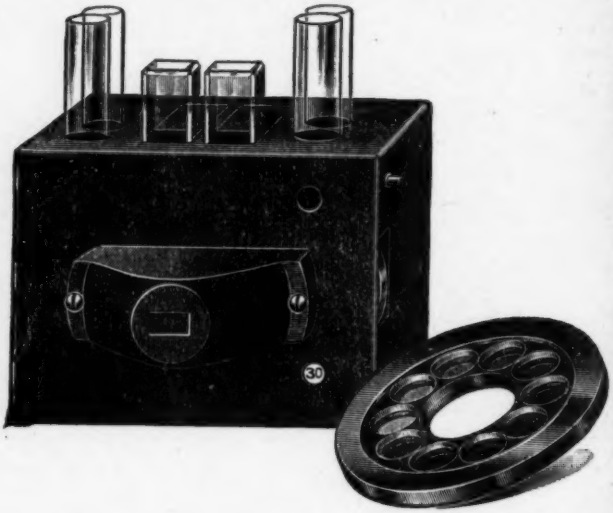

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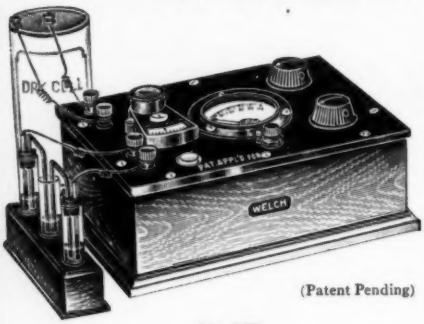

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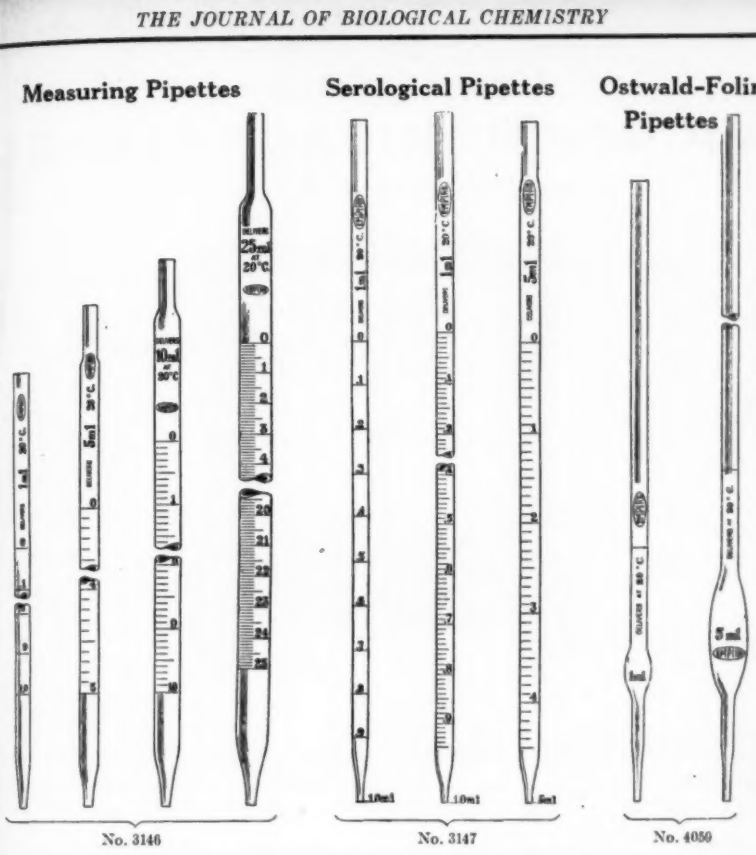

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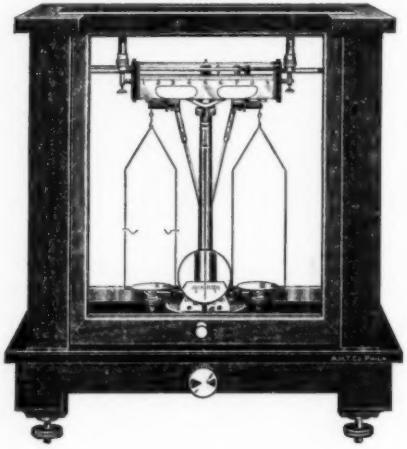

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CORRECTIONS.

On page 515, Vol. lxxvi, No. 2, February, 1928, Table II, fifth entry, last three columns, read *−40,600, 95,900, 14,700* for *−37,800, 93,100, 17,500* respectively.

On page 519, line 18 read

$$[\alpha]_D^{20} = \frac{-2.90° \times 100}{1 \times 5.00} = -58.0°$$

for

$$[\alpha]_D^{20} = \frac{-1.64° \times 100}{1 \times 2.00} = -82.0°$$

ents not involved in producing the prompt acceleration of blood formation (4). In the absence of any hypothesis regarding the

* The expenses of this investigation have been met in part by grants from the De Lamar Research Fund of the Harvard Medical School, and in part from the Farnsworth Fund.

† It is a pleasure to acknowledge the cooperation of Dr. William P. Murphy, of the Peter Bent Brigham Hospital, of Dr. James H. Means and Dr. Wyman Richardson of the Massachusetts General Hospital, of Dr. William B. Castle of the Thorndike Memorial Laboratory of the Boston City Hospital, and of Dr. Herman A. Lawson and Dr. Franklin R. Miller of the Collis P. Huntington Memorial Hospital of Harvard University.

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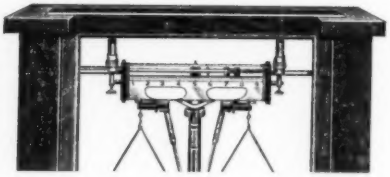

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(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, and the Medical Services of the Collis P. Huntington Memorial Hospital of Harvard University, the Peter Bent Brigham Hospital, and the Massachusetts General Hospital, Boston.)†

(Received for publication, January 23, 1928.)

INTRODUCTION.

Advances in practice, resting upon observation and experience, often precede exact knowledge of the underlying reactions and mechanisms of natural phenomena. Such an advance was the demonstration by Minot and Murphy (12–14) that the feeding of large amounts of liver is followed by an increase of concentration of red cells in the blood in cases of pernicious anemia. This discovery raises the question of the nature of the substance, or substances, which are thus effective, as well as that of the character of the disturbed physiological processes which are modified by the addition of liver or kidney to the diet.

Accordingly the chemical dissection of the liver has been undertaken as a means of eliminating, one by one, those of its constituents not involved in producing the prompt acceleration of blood formation (4). In the absence of any hypothesis regarding the

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nature of the active principle the empirical procedure, now to be described, was resorted to. The decision was made always to divide the liver into the smallest possible number of fractions, to discover which one of these fractions was most active, and to continue by subdividing the active fraction.

The facts that were relied upon to indicate whether or not the active constituent, or constituents, of the liver had been extracted, were furnished by clinical observations. Particularly important is the observation that the feeding of adequate amounts of liver to patients with pernicious anemia in relapse is regularly followed by a prompt and marked rise in the concentration of red blood cells, for the most part distinguished by their reticulation (15). Although our preparations had to be fed to patients with pernicious anemia in order to determine whether or not they were effective, the results were rarely ambiguous, and proved to be of a semiquantitative nature. This was due, in part, to the experience previously gained by Minot and Murphy while treating pernicious anemia with whole liver. These earlier observations on the increasing concentration of red blood cells in blood, together with those made while feeding the extracts, lend themselves to quantitative formulation, since the reactions initiated by the active principle take place in a very large number of cells. Many processes not yet entirely understood are involved; they occur within the human body, and they are occasionally modified by the state of the patient and by complications of the disease. Thus a certain variability is introduced into the results. The body must be considered the environment in which the reactions with which we are concerned take place. These reactions result in the rapid and prompt formation of several hundred thousand red blood cells per c.mm. of blood, following the ingestion of a small amount of material. Although the mass of the material is small, it contains, of course, a large number of molecules of the active substance. Because of the large numbers of cells and molecules involved there is reason to believe that the results may be successfully subjected to statistical treatment.

Erythrocyte and Reticulocyte Response.

In recording the response of the patient's red blood corpuscles in quantitative terms the following algebraic symbols have been

employed. The concentration of red blood corpuscles, or erythrocytes, has been designated by the symbol E, expressed as millions per c.mm. E_0 represents the number of red blood corpuscles per c.mm. in the capillary blood of a patient before the administration of a fraction of liver. This figure was obtained from a series of observations. The difference, $(E_{10} - E_0)$, thus represents the net increase in the number of red blood corpuscles per c.mm. in the capillary blood during the first 10 days of feeding.

The percentage of erythrocytes that are reticulated corpuscles is designated r. The concentration of reticulocytes present in the blood on any day is given by the product Er. In so far as there has been no destruction of red blood cells, no change in the distribution of body fluids, no maturation of reticulocytes to adult red blood cells, and no entrance into the circulation of non-reticulated erythrocytes, it must follow that:

$$Er = (E - E_0) \tag{1}$$

In the records of the cases presented, and in their graphical representation, both the quantities Er and $(E - E_0)$ are often given.

Within the first few days after feeding of the extract the quantity $(E - E_0)$ frequently became negative, indicating either an increase in volume of the fluid at the point of removal or a destruction of red blood cells. This period rarely exceeded the first 4 or 5 days following the feeding of a potent fraction. Thereafter both $(E - E_0)$ and Er increased. The amount of the increase in total erythrocytes continued at a comparable rate, but the number of reticulocytes diminished. Apparently maturation of the reticulocytes that first appeared had begun in the blood, and most of the newly formed erythrocytes had had time to mature beyond the reticulated stage before their extrusion from the bone marrow. The largest number of reticulocytes appeared in the blood in from 5 to 10 days, depending in part upon the potency of the extract. Within a subsequent 5 to 10 day period the reticulocytes had generally decreased to a low level in the blood stream. It would appear to be a fair deduction from these observations that the reticulocytes in the blood did not lose the reticular material, upon which their vital staining depends, for from 5 to 10 days. The rate at which reticulocytes lose the material that identifies them may however be a

function of the concentration in the blood of the active principle effective in pernicious anemia.

The algebraic notation adopted in this paper had not been devised when these observations were begun. Accordingly the importance of daily measurements, not only of the percentage of reticulocytes, r, but also of the concentration of erythrocytes, E, was not understood. The percentage of reticulocytes is, however, as elsewhere demonstrated (11, 14), largely a function of the level of the red blood cell count. The number of reticulocytes is more nearly independent of the red blood cell concentration, revealing the potency of the extract, although this quantity still shows a definite variation with the red blood cell level, and a lesser variation between cases with the same erythrocyte concentration. The clinical experience derived from the feeding of liver extract is discussed in another paper (11).

The erythrocyte level has occasionally been estimated on days when it was not measured, by graphical interpolation, in order to permit the calculation of the number of reticulocytes, Er. The error in estimating the percentage of reticulocytes is usually not greater than 3 per cent, whereas the error in counting the number of red blood cells is frequently as great as 150,000 per c. mm. or more. The number of erythrocytes formed within the first few days generally ranges from 300,000 to 500,000 per c. mm., whereas the percentage of reticulocytes may increase from a fraction of 1 per cent to 50 per cent, or more. It follows that a slight error in the estimation of E by interpolation will introduce no great error in the product Er.

Another method may be suggested for estimating the erythrocyte concentration, within the first few days, from the reticulocyte concentration. Provided the only source of the increase in erythrocytes is through the formation of reticulocytes, it follows from equation (1) that:

$$E = E_0 + Er \tag{2}$$

Transposing, we have the relation:

$$E = \frac{E_0}{(1 - r)} \tag{3}$$

and

$$Er = \frac{E_0\, r}{(1 - r)} \tag{4}$$

Combining equations (1) and (4) we obtain:

$$Er = \frac{E_0\, r}{(1 - r)} = E - E_0 \tag{5}$$

These conditions can only be expected to obtain provided there has been no significant destruction of red blood cells, nor change in their concentration as a result of redistribution of body fluids. In fact not only must these conditions obtain, but during the length of time that these three equations yield identical results there can have been no appreciable change of the reticulocytes to adult cells in the blood, nor delivery of adult forms from the bone marrow. In the majority of cases these quantities have not been identical, but their relations to each other are none the less significant.

Accurate information as to whether an extract was effective was usually revealed by the reticulocyte count within a week. Information of comparable accuracy could not have been gleaned from the erythrocyte count in less than from 2 to 3 weeks.

The change in the concentration of reticulocytes and of erythrocytes in the blood of patients with pernicious anemia that were fed various fractions of beef or pig liver may now be considered. These responses revealed something of the nature of the disturbed reactions in the blood in pernicious anemia, whereas the chemical fractionation yielded information regarding the specific substances which initiated the responses.

Chemical Fractionation.

Insoluble Residue (A).—The first fractionation of the liver was attempted in collaboration with Dr. John F. Fulton. It was decided as a beginning to isolate the characteristic copious casein-like liver protein. This protein is soluble in alkaline solution, but insoluble in the neighborhood of pH 5. Accordingly fresh minced beef liver was rendered alkaline by the addition of sodium hydroxide. Since Bradley (3) has shown that the liver enzymes are rela-

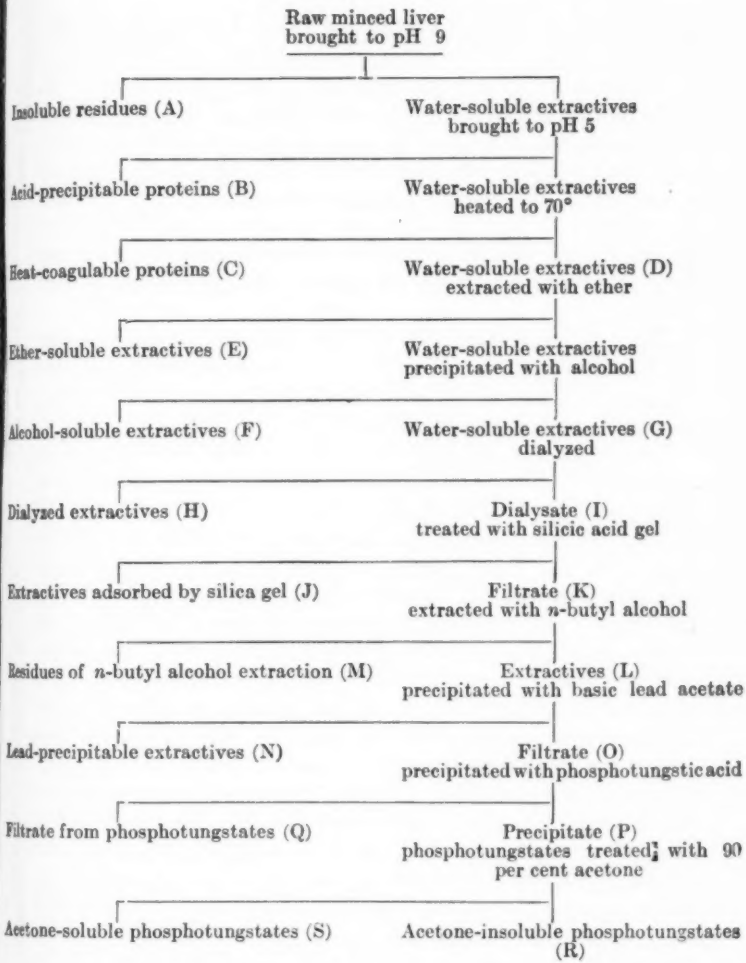
tively inactive at pH 9, enough alkali was added to bring the tissue to this reaction, as it seemed wise to effect a separation under such conditions that the liver would be as little altered as possible during the procedure. The soluble extractives were then separated from the insoluble residues. These fractions and those subsequently prepared have for convenience been designated by letters essentially in the order in which they were attempted. Their relation to each other is represented in the accompanying diagram. The procedures represented are not equally useful. Some are now rarely used, and all have not so far been employed in any one preparation.

The residues (A) were subsequently fed, together with the acid-precipitable proteins (B), to a patient with pernicious anemia, without measurably influencing blood formation. The fraction designated (A) was thus shown not to contain any significant amount of the active principle. Besides connective tissue this residue contains such insoluble proteins, fats, and carbohydrates as are present in liver.

Characteristic Liver Protein (*B*).—The characteristic soluble liver protein represents the most abundant constituent of the filtrate. The liver protein that dissolved with the soluble extractives at pH 9 was next precipitated in the neighborhood of its isoelectric point. This was at first accomplished by adjusting the reaction nearly to pH 5 by hydrochloric acid, and at a later date by sulfuric acid. The copious flocculent precipitate that separates under these conditions may be removed by sedimentation, centrifugation, or filtration from the other soluble extractives. All three methods have been employed at one time or another. The protein precipitate was always repeatedly washed. In one preparation it was further purified by redissolving with alkali and reprecipitating with acid.

This protein, fraction (B), was fed to Patient 1 for 13 days, with negative results. A few days later when he was given 200 gm. of raw liver pulp daily he responded in the customary manner with a prompt increase in the concentration of reticulocytes and of erythrocytes.

Since the active principle was in neither fraction, (A) or (B), it was later decided to eliminate the preliminary alkaline extraction. In subsequent preparations the minced liver was immediately



brought to the apparent isoelectric point of the liver protein by means of acid, and the water-soluble substances repeatedly extracted from the copious precipitate consisting of certain proteins, carbohydrates, and lipoids of the liver. Approximately

40 cc. of normal acid sufficed to bring 1 kilo of minced liver to the desired reaction. The resulting precipitate was repeatedly washed by sedimentation and centrifugation. This process was carried out in the cold with the result that hydrolysis of carbohydrates and proteins was inhibited. In one case the precipitate, (A + B), after five successive extractions with 3 volumes of water, was fed to a patient. The benefit resulting to the patient and the increase of reticulocytes were so slight as to indicate that practically complete extraction of the active principle from the liver was possible. In practice it has not always been expedient to extract so completely.

Although the results with the liver protein (B) and with the combined precipitates (A + B) were negative, they indicated that large portions of the constituents of liver are inert, and that the active principle was soluble in water.

Heat-Coagulable Proteins (*C*).—The extractives soluble at slightly acid reactions still contain protein which coagulates upon being heated to from 60–70°. These proteins appear to be derived in large part from the blood contained in the liver, rather than from the tissue itself, and represent the albumins and globulins of the blood, together with any similar constituents of the liver. Accordingly their study was postponed until after that of the essentially protein-free filtrate obtained from the coagulum.

Water-Soluble Extractives (*D*).—The non-protein extractives dissolved in the filtrate from (C) had reached so large a volume that concentration was resorted to. The wash waters of the second preparation were therefore concentrated on a water bath. A part of the material was rendered insoluble during this process, but as much as dissolved was fed to a patient. The record of this case is in part presented in Table I. Feeding of this extract was promptly followed by increase of the reticulocyte concentration, though to a smaller extent than when a maximal amount of potent material is given. A second and much larger reticulocyte response followed the daily feeding of liver pulp, clearly indicating that the extract had been weak.

This first response, though incomplete, led to the belief that the active principle had been extracted, but had been largely destroyed during the prolonged concentration at high temperature. Accordingly a vacuum still was constructed and employed in concentrat-

ing the water-soluble extractives of Preparations IV to XVII. The temperature was maintained at 60°, since it had been shown that this temperature did not rapidly destroy the active principle. The resulting concentrate (D) has been prepared as a solution, as a viscous syrup containing but 25 per cent of water, and as a dry powder. It was fed to Patients 3, 4, and 5, with the results recorded in Table II.

TABLE I.

Patient 2.

Days upon fraction.	Designation of fraction.	Amount of fraction given.	Reticulocytes. (*r*)	Concentration of erythrocytes. (*E*)	Concentration of reticulocytes. (*Er*)
		gm.	*per cent*	*millions per c. mm.*	*millions per c. mm.*
0			2.1	1.24	0.026
1	II D	9.45	3.0	1.41	0.042
2		9.45	1.9	1.28	0.024
3		9.45	1.0	1.10	0.011
4		9.45	1.0	1.29	0.013
5		9.45	0.3	1.40	0.004
6		9.45	1.5	1.14	0.017
7		9.45	2.9	1.37	0.040
8		9.45	5.6	1.28	0.072
9		9.45	10.1	1.38	0.139
10		9.45	16.0		0.221
11		18.90	7.2	1.38	0.099
12		18.90	3.1	1.47	0.046
13		18.90	6.6		0.091
14		18.90	2.1	1.23	0.026
15		18.90	2.8	1.32	0.037
16		*			

* Patient given 200 gm. of raw liver pulp.

Each of these three patients received each day the extract from approximately 400 gm. of liver. The laboratory had not yet been adequately equipped for the preparation of extract, and accordingly but an uncertain amount of a weak extract was fed to the first of the three patients (Patient 3) who received extract (D), from the time when Preparation III D was finished until Preparation IV D was ready. This irregularity in the administration of the extract is reflected in the reticulocyte response, and graphically represented in Fig. 1. After taking a potent extract for 4 days,

TABLE II.

Days upon fraction.	Designation of fraction.	Amount of fraction given.	Reticulocytes. (r)	Concentration of erythrocytes. (E)	Concentration of reticulocytes measured. (Er)	Concentration of reticulocytes estimated. $\left(\frac{Er}{1-r}\right)$	Change in concentration of erythrocytes. ($E - E_0$)
Patient 3.							
		gm.	*per cent*	*millions per c. mm.*	*millions per c. mm.*	*millions per c. mm.*	*millions per c. mm.*
0			2.5	1.83	0.046	0.047	
1	III D	8.10	1.1			0.020	
2		8.10	1.6	1.88	0.030	0.030	0.05
3		8.10	2.0			0.037	
4		8.10	0.8	(2.33)*	0.019	0.015	
5	IV D	†	1.0		0.023	0.018	
6		†	1.5		0.035	0.028	
7		†	4.2		0.103	0.080	
8		15.72	5.6	(2.47)	0.138	0.109	
9		15.72	4.9		0.121	0.094	
10		15.72	5.6		0.134	0.109	
11		15.72	9.0		0.207	0.181	
12		15.72	12.4	2.20	0.273	0.259	0.37
13		15.72	11.6		0.267		
14		15.72	7.8		0.187		
15		15.72	6.5		0.163		
16	V D	16.54	6.7		0.174		
18		16.54	1.3	2.88	0.037		1.05
20		16.54	2.1	2.95	0.062		1.12
22		16.54	0.5		0.015		
23	VI D	11.86					
24		11.86		2.98			1.15
25		11.86					
26		11.86	0.1	3.06	0.003		1.23
30	VII D	19.42	1.0	3.40	0.034		1.57
33		19.42		3.50			1.67
38	VIII D	14.06		3.89			2.06
40		14.06		3.80			1.97
Patient 4.							
0			0.7	2.75	0.019	0.019	
1	III D	10.8	0.3	2.68	0.008	0.008	−0.07
2		10.8	0.7			0.019	
3		13.5	0.8	(2.93)	0.023	0.022	
4	IV D	‡	2.2			0.062	
5		‡	1.3	2.74	0.036	0.036	−0.01
6		‡	2.9	2.88	0.084	0.082	0.13
7		‡	5.4		0.156	0.156	
8		15.72	7.6	2.87	0.218	0.226	0.12
9		15.72	8.3		0.238	0.249	

TABLE II—*Concluded.*

Days upon fraction.	Designation of fraction.	Amount of fraction given.	Reticulocytes. (r)	Concentration of erythrocytes. (E)	Concentration of reticulocytes measured. (Er)	Concentration of reticulocytes estimated. $\left(\frac{E\sigma}{1-\sigma}\right)$	Change in concentration of erythrocytes. ($E - E_0$)
Patient 4.—Continued.							
		gm.	*per cent*	*millions per c. mm.*	*millions per c. mm.*	*millions per c. mm.*	*millions per c. mm.*
10		12.58	4.5	2.99	0.135	0.130	0.24
11		12.58	4.3		0.131	0.124	
12		12.58	2.3		0.072	0.065	
13		§	4.2		0.137		
14	V D	16.54	3.5	3.54	0.124		0.79
16		16.54	1.6	3.69	0.059		0.94
18		16.54	0.7	3.53	0.025		0.78
21		§	0.8	(3.04)	0.024		
22	VI D	11.86		(3.20)			
23		11.86	0.8				
24		11.86		3.84			1.09
26		11.86	0.3	(3.53)	0.011		
29	VII D	19.42	0.1	4.31	0.004		1.56
31		19.42	0.2	4.28	0.009		1.53
36		‖		4.48			1.73
Patient 5.							
0			0.9	1.58	0.014	0.014	
1	IV D	15.72	1.7		0.027	0.027	
2		15.72	0.8	1.58	0.013	0.013	
3		23.58	1.9		0.030	0.031	
4		23.58	1.5	1.59	0.024	0.024	0.01
5		23.58	3.0		0.048	0.049	
6		23.58	5.0		0.080	0.083	
7		15.72	11.0		0.169	0.195	
8	V D	24.81	13.8	1.54	0.213	0.253	−0.04
9		24.81	14.0			0.257	
10		24.81	9.9				
11		24.81	9.5	2.48	0.236		0.90
12		16.54	8.9		0.231		
13		8.27	3.9		0.105		
14		¶	6.2		0.174		
15			4.2	2.93	0.123		1.35

* Erythrocyte counts that were not used in the calculation of $(E - E_0)$ are printed in parentheses.

† An uncertain amount was given for 3 days of the first part of Preparation IV D.

‡ Patient given 200 cc. per day of first part of Preparation IV D.

§ None administered.

‖ Patient given 180 gm. of raw liver pulp.

¶ Patient given 240 gm. of raw liver pulp.

and at a time when the reticulocytes were increasing in the peripheral blood, this patient received a weak extract for 3 days. The reticulocytes continued to increase in number for approximately 4 more days, but the response then lapsed until 2 days after the administration of the new and more potent extract, Preparation IV D. Thereafter the number of reticulocytes further increased until the peak of their rise was reached on the 12th day after the extract was first given. At that time there were 273,000 reticulated red blood cells in each c.mm. of blood. After the 13th day their number progressively diminished, and after

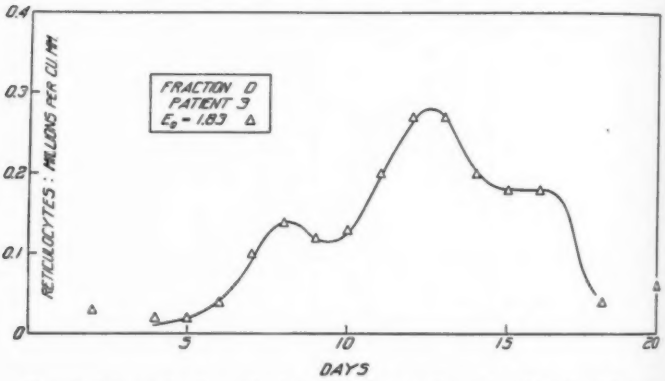


FIG. 1. Change in the reticulocyte concentration, *Er*, of Patient 3 upon varying amounts of fraction (D).

another period of about 8 days returned to normal. By this time newly formed erythrocytes, without reticulum, had begun to appear in the blood, which contained 1 million more red blood cells per c.mm. than at the beginning of feeding. The net increase in the total number of erythrocytes ($E - E_0$) of Patient 3, and of certain others, is graphically represented in Fig. 3. In Patient 3 after 40 days the red blood cell concentration had been increased by nearly 2 million per c.mm. That is to say, the number of corpuscles had more than doubled, and during this time the hemoglobin percentage had nearly doubled.

The next two patients (Nos. 4 and 5) who received the (D) type of extract responded slightly more rapidly than the first.

Although their red blood cell counts differed by approximately 1 million cells per c.mm. before and during the first days of feeding, the absolute numbers of reticulocytes at the peak of their rise were almost identical. Each had in his blood approximately 240,000 reticulocytes per c.mm. by the 9th day. Thereafter the number of these cells in the peripheral blood diminished, though more rapidly in one case than in the other. Patient 4 received for 36 days the successive extracts of the (D) type that were prepared. At the end of this time the number of red blood cells had increased 1.73 millions per c.mm. and had become almost normal (Fig. 3). The other patient (No. 5), with a much lower red blood cell count, was fed the fraction for only 13 days, and the red blood cell count

TABLE III.

Solubility of Fraction (D) in Organic Solvents.

Solvents in order employed.	Per cent of fraction extracted from 2.098 gm. Preparation VD.	Solvents in order employed.	Per cent of fraction extracted from 1.872 gm. Preparation VD.
Ether.	1.4	Ether.	1.0
Acetone.	6.2	Acetone.	7.9
Ethyl alcohol.	33.6	Ethyl alcohol.	34.4
n-Butyl alcohol.	30.9	Glacial acetic acid.	30.1
Pyridine.	0.8	Pyridine.	1.3
Total extracted.	72.9	Total extracted.	74.7

had increased by over 1 million cells per c.mm. The feeding of the fraction was then discontinued for lack of material, but the erythrocytes continued to rise to normal upon whole liver.

These results indicated that the active principle of the liver, effective in pernicious anemia, had been extracted and was contained in fraction (D). Of the solids in this crude extract approximately 20 per cent were inorganic substances, recoverable after ashing the material. From 5 to 8 per cent consisted of nitrogen, present neither as protein nor as ammonia.

During the time when patients were being fed fraction (D), experiments intended to suggest a method of further purifying the extract were in progress. To this end the solubility of fraction

(D) was studied in a large number of organic solvents. The results of two such experiments[1] are recorded in Table III.

Ether-Soluble Extractives (*E*).—The extraction of the liver with water left the largest part of the lecithin and other lipoids undissolved in fraction (A). But from 1 to 3 per cent of the different preparations of fraction (D) was ether-soluble. None the less it seemed desirable to test one patient upon a fraction from which all the ether-soluble material had been extracted. The reticulocyte response of this patient although smaller than that obtained with the (D) type of extract, was, nevertheless, definite. The active principle effective in pernicious anemia appeared to be no more of a lipoidal than of a protein nature.

Alcohol-Soluble Extractives (*F*).—Approximately 7 per cent of the solids in fraction (D) were found to be soluble in acetone (Table III). Over 30 per cent dissolved in ethyl alcohol. Moreover the material dissolved by acetone was also soluble in ethyl alcohol. Accordingly fraction (D) was further purified by the elimination of the alcohol-soluble extractives (F).[2] This was first accomplished by pouring a concentrated aqueous solution of extract (D) into such an amount of absolute alcohol as to render the final alcohol concentration approximately 95 per cent by volume. The alcohol was maintained in violent agitation by a mechanical stirrer while the extract was slowly added. As a result the precipitate that appeared when the syrup was poured into the absolute alcohol was finely divided. After some time had elapsed the stirring was stopped and the precipitate removed by sedimentation and filtration. The precipitate was washed with a small volume of alcohol and the wash waters added to the filtrate (F) containing the alcohol-soluble extractives.

The concentrated alcoholic extract (F) has both a sharp taste and odor. It contains certain of the nitrogenous bases in the liver, but of those that are alcohol-soluble, such as choline and histamine, all are probably not extracted by this fractionation (2). Certain carbohydrates also dissolve and are removed in fraction (F).

[1] These experiments were conducted by Mrs. F. C. Sargent.

[2] The liver fraction fed to patients with pellagra by Voegtlin (20) was alcohol-soluble and was extracted directly from the dried liver by this solvent.

Fraction (F) was fed to one patient without producing beneficial results. At the same time the alcohol-precipitable material (G) was fed to a patient who responded characteristically with an increased concentration of reticulated red blood cells.

Alcohol-Precipitable Extractives (G).—The precipitate that appeared when fraction (D) was poured into sufficient alcohol to render the final concentration 95 per cent by volume, contained the active principle. In continuing the preparation this precipitate was generally not completely dissolved. Instead the type of fractionation employed by Osborne and Wakeman (17) in the concentration of water-soluble vitamin, was attempted. It was found that most, if not all, of the chemical substance effective in pernicious anemia was dissolved if sufficient water was added to the precipitate to render the concentration of alcohol 70 per cent. A large amount of inert material remained undissolved in this alcohol mixture, and thus a substantial concentration was achieved. The precipitate was repeatedly washed with 70 per cent alcohol to remove occluded material, and the washings were added to the solution containing the active principle.

Although fraction (G) was first successfully prepared in this manner, it was later found convenient to reverse the procedure, in order to obtain a dry powder as the final product. In the more recent preparations only enough alcohol was added to fraction (D) to render the alcoholic concentration 70 per cent. The resulting precipitate was repeatedly washed with 70 per cent alcohol and the combined filtrates concentrated *in vacuo*. This concentrate was then poured, in the manner previously described, into sufficient absolute alcohol to render the final concentration at least 95 per cent by volume. The resulting precipitate was collected upon a Buchner funnel, washed with 95 per cent alcohol, then with absolute alcohol, and finally with anhydrous ether. The precipitate, which proved to be very hygroscopic, was continuously covered with alcohol or ether until it had been transferred to a desiccator.

Fraction (G) appeared to be at least as effective as (D), or as whole liver, in the treatment of pernicious anemia (Table IV). The first patient (Patient 8) to whom fraction (G) was fed responded with a very prompt and marked increase of reticulocytes. This individual was given extract derived from ap-

TABLE IV.

Days upon fraction.	Designation of fraction.	Amount of fraction given.	Reticulocytes. (r)	Concentration of erythrocytes. (E)	Concentration of reticulocytes measured. (Er)	Concentration of reticulocytes estimated. $\left(\frac{E_0 r}{1-r}\right)$	Change in concentration of erythrocytes. ($E - E_0$)
Patient 8.							
		gm.	*per cent*	*millions per c. mm.*	*millions per c. mm.*	*millions per c. mm.*	*millions per c. mm.*
0			0.4	1.45	0.006	0.006	
1	VII G	9.79	0.2			0.003	
2		9.79	0.4			0.006	
3		9.79	2.1			0.031	
4		9.79	1.6	1.44	0.023	0.024	−0.01
5		9.79	4.6			0.070	
6		3.92	6.0			0.093	
7	VIII G_1	5.72	11.7	1.65	0.193	0.192	0.20
8		5.72	13.2			0.221	
9		9.16	10.5			0.170	
10		9.16	17.9	1.81	0.324	0.316	0.36
11		9.16	15.9				
12	VIII G_2	9.06	16.4				
13		9.06	14.8	2.00	0.296		0.55
14		9.06	13.6				
15		9.06	11.7				
16		9.06	7.1				
17		9.06	4.5	2.19	0.099		0.74
18		9.06	2.8				
20	X G	7.60	2.5	2.46	0.062		1.01
24		7.60	1.3	2.47	0.032		1.02
27	XI G	13.90	0.4	2.74	0.011		1.29
31		*	0.2	2.83	0.006		1.38
Patient 10.							
0			0.1	0.55	0.001	0.001	
1	VIII G_1	9.16	0.8	0.62	0.005	0.004	0.07
2		9.16	0.8			0.004	
3		9.16	6.6	0.48	0.032	0.039	−0.07
4	VIII G_2	14.48	23.2			0.166	
5		14.48	41.4	0.68	0.282	0.389	0.13
6		14.48	30.0			0.236	
7		14.48	35.8			0.307	
8		14.48	14.3	1.50	0.215		0.95
10		7.24	9.6	1.61	0.155		1.06
11	X G	7.60					
12		7.60	4.6	1.80	0.083		1.25
15		7.60	1.0	2.21	0.022		1.66
17		7.60	3.0				
18	XI G	13.90		2.40			1.85
19		13.90	1.8				
22		*	0.4	2.94	0.012		2.39

* Patient given raw liver pulp.

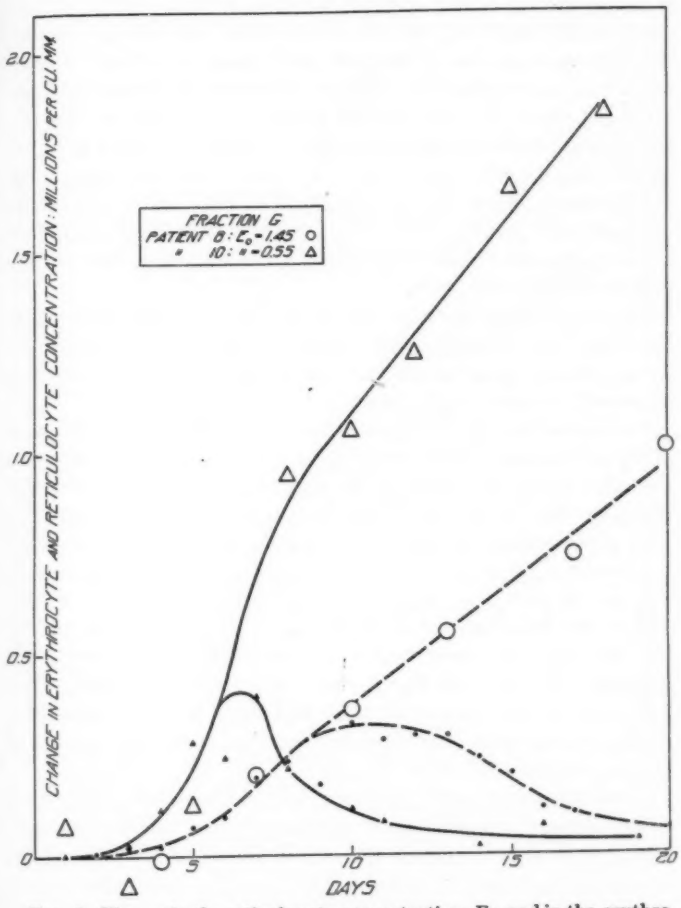


FIG. 2. Change in the reticulocyte concentration, Er, and in the erythrocyte concentration ($E - E_0$) of Patients 8 and 10 upon different amounts of fraction (G). The errors in determining reticulocyte concentration are far smaller and smaller solid symbols are therefore employed in designating this quantity, whereas large hollow symbols are employed in designating the erythrocyte concentration.

proximately the same amount of liver as the patients given fraction (D), and the rates of reticulocyte, and of erythrocyte, increase under the two treatments were almost identical. In this patient given fraction (G) the concentration of reticulocytes per c. mm. of blood, *Er*, was slightly greater at the peak of the rise than in the patients given fraction (D), but the red blood cell level before feeding (E_0) was lower (11, 15). The net increase in erythrocytes, ($E - E_0$), is compared in Fig. 3 with the results obtained with fraction (D). The concentration of red blood cells in the peripheral blood increased by 1 million per c. mm. in the first 20 days in this case.

The erythrocyte increase during the first 10 days appears to have been due entirely to the formation of reticulocytes (Fig. 2). These results thus appear to conform to the ideal conditions expressed by equations (1) and (5).

The second patient (Patient 10) to receive fraction (G) had only about 0.5 million red blood cells per c. mm. and was in a comatose condition when the material was first administered. Accordingly it was decided to feed larger amounts of the extract (G) than had been given to the first case. The more rapid reticulocyte response that resulted (Fig. 2), depended in part upon the larger amount fed, but in part, also, upon the lower erythrocyte level. Within 7 days 300,000 reticulocytes per c. mm. were in the blood, and by the 8th day the concentration of erythrocytes had more than doubled. By the 15th day the net increase in erythrocytes was 1.66 and by the 22nd day, 2.4 million per c. mm. Since then similar results have been regularly obtained when this amount of the fraction has been given (Fig. 3).

Although fraction (G) has been prepared free of iron, of proteins, and of lipoids, and although it was considered satisfactory from a therapeutic standpoint, it was still a relatively crude extract judged either from the chemical or from the physiological standpoint. The larger molecules had for the most part been removed, but the ash still represented nearly 20 per cent of the solids. Evidence, though inconclusive, suggested that the molecule of the active principle is of dialyzable size. After electrodialysis the fraction retained between parchment membranes, designated (H), proved inactive when fed to a patient. Another patient had an unexpectedly weak reticulocyte response when fed extract after

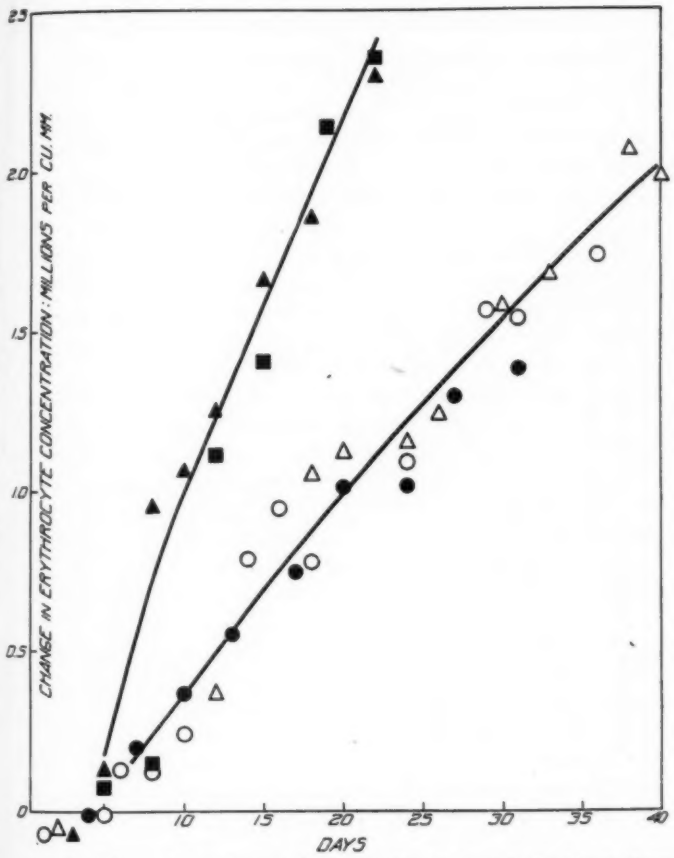


FIG. 3. Change in erythrocyte concentration ($E - E_0$) of patients given different amounts of extract.

△ Patient 3, E_0 = 1.83 fraction (D) derived from about 400 gm. of liver.
○ " 4, " = 2.75 " " " " " 400 " " "
● " 8, " = 1.45 " (G) " " " 400 " " "
▲ " 10, " = 0.55 " " " " " 600 " " "
■ " = 1.22 Liver Extract No. 343 derived from about 600 gm. of liver.

ultrafiltration through a collodion membrane at a low temperature (I). The observation that the substance is effective when given

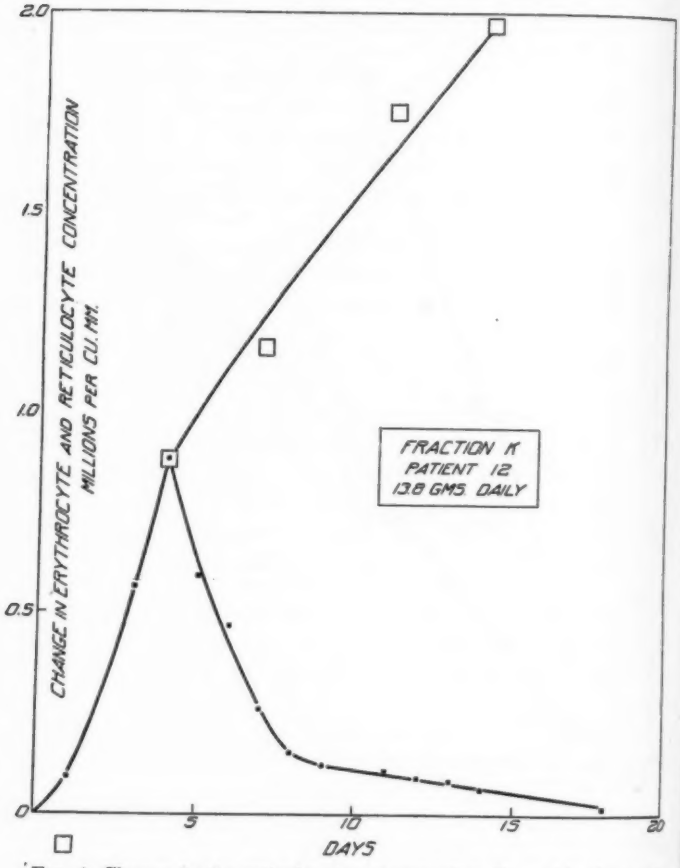


FIG. 4. Change in the reticulocyte concentration, Er, and in the erythrocyte concentration ($E - E_0$) of Patient 12, $E_0 = 1.26$.

by mouth, suggested that the dimensions of the molecule are not such as to interfere with its absorption.

Fraction (G) was free of the blood sugar-reducing substances of

liver (4, 16), but it still accelerated the denervated heart. Although free from such anticoagulating effects as might be expected were heparin present, it appreciably lowered blood pressure when injected intravenously in a cat.[3] Indeed certain of the methods employed in this investigation have not been dissimilar to those described for the extraction and purification of blood pressure-reducing substances in liver (8, 10). The active principle effective in pernicious anemia is for the most part not precipitated, however, by the same reagent concentrations.

From the solubility of the material effective in pernicious anemia and that of known vitamins it became evident that the liver extract was free of those vitamins that are lipoid-soluble, but that it might still contain a water-soluble vitamin. The instability and the other properties of vitamin C appeared to exclude its consideration. With the possibility in mind that so called "vitamin B" might be responsible for the effectiveness of liver extract in the treatment of pernicious anemia, the method of concentration of vitamin B described by Levene and van der Hoeven (9) was attempted. The extract was treated with silicic acid gel at pH 5.0. The active principle was not adsorbed by the gel (J), but remained in the filtrate, designated (K), which when fed to Patient 12 produced the results graphically represented in Fig. 4. The gel however was inactive.

This kind of negative evidence concerning so called vitamin B was not considered satisfactory. Large amounts of different sources of the antineuritic and the pellagra-preventive factors were therefore fed to three patients with pernicious anemia. The first individual was given about 1000 gm. of yeast-cake in 8 days. The second received 407 gm. of a dried aqueous yeast extract in 8 days.[4] In these two patients there occurred in 15 days no

[3] We are indebted to Dr. Paul M. Harmon for testing the blood pressure-reducing effects of the various extracts. In the course of these observations acceleration of the denervated heart was observed.

[4] We are indebted to Dr. Joseph Goldberger for supplying us with this preparation—a commercial dried aqueous yeast extract. This material has been shown by Goldberger, Wheeler, Lillie, and Rogers (6) to be potent in but a few gm. a day as a preventive and curative agent in pellagra and experimental black tongue of dogs. Thus the pellagra-preventive factor appears not to be identical with the material which is effective in pernicious anemia.

change in their blood or general condition, but they later responded, one to liver, and one to liver extract. The third received 80 gm. of a vitamin extract derived from over 9 kilos of yeast, and 720 cc. of an extract derived from over 7 kilos of wheat embryo in 8 days. This patient showed some slight improvement, but not such as would be expected with even moderate amounts of liver extract. None the less the effects of different sources of the factors of so called vitamin B are being further investigated.

Butyl Alcohol Extractives (*L*).—Certain of the constituents of (G) can be extracted by means of such organic solvents as normal butyl alcohol, pyridine, and glacial acetic acid (Table III). The attempt was made at this point in the investigation to effect a separation by means of normal butyl alcohol. The type of Kutscher-Steudel apparatus described by Dakin (5) was employed under reduced pressure, and the extractives, (L), freed of the butyl alcohol by means of ether, were given to a single patient. A slight reticulocyte rise followed, but the experiment could not be continued at the time because sufficient extract was not available. The results suggested that the active principle had been extracted by means of butyl alcohol, though not, perhaps, without a certain amount of loss of potency. Because of the inadequate facilities available for the preparation of fractions, this process was abandoned in favor of those that were beginning to yield more striking results.

Lead-Precipitable Extractives (*N*).—Slightly more than half the constituents of fraction (G) were precipitable by lead. Sufficient alkali, either barium hydroxide or sodium hydroxide, depending upon whether or not it was desired to keep the salt content of the filtrate at a low level, was added to bring fraction (G) to approximately pH 8.0. Basic lead acetate was then added until precipitation was complete. The basic lead acetate yielded a copious precipitate which readily separated upon centrifugation or filtration. The precipitate, (N), was repeatedly washed with small quantities of water and the wash waters added to the centrifugate or filtrate (O). Basic lead acetate, a generally useful precipitant, was also employed by Best, Dale, Dudley, and Thorpe in their study of "The Nature of the Vaso-Dilator Constituents of Certain Tissue Extracts" (2).

The precipitate was slightly acidified, and the lead and barium

were removed as sulfate and sulfide.[5] This fraction (N) was then given to a patient with pernicious anemia, without, however, producing any significant reticulocyte response.

Lead Filtrate (*O*).—The filtrate from the lead precipitate was slightly acidified, generally with sulfuric acid, and the lead removed as sulfate and sulfide. The lead-free filtrate (O) was concentrated under reduced pressure to the desired volume, and

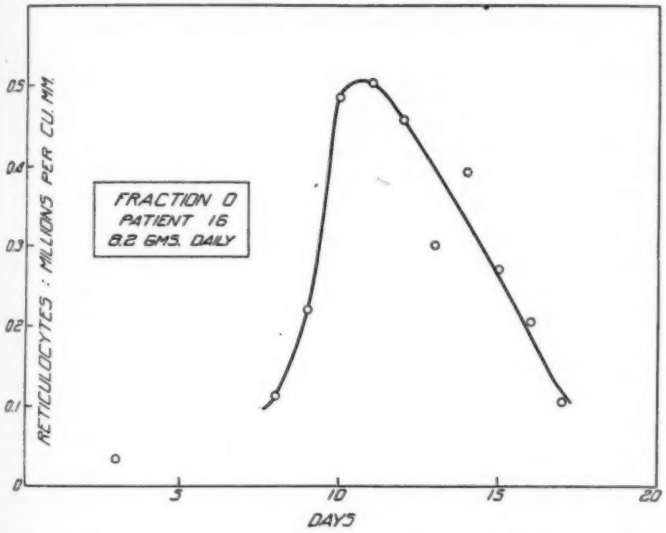


FIG. 5. Change in the reticulocyte concentration, *Er*, of Patient 16, E_0 = 1.73.

administered to Patient 16 with the results graphically represented in Fig. 5. In the belief that a part of the active material might have been lost in the copious precipitates involved in this purification, the extract derived from 1 kilo of liver a day was fed. Even so, but 8.2 gm. of solids were given each day, and within 11 days 0.5 million reticulated red blood cells per c.mm. of blood were present. The diminishing erythrocyte count of the patient prior

[5] We are indebted to Dr. Reid Hunt for testing these, and subsequent fractions, for freedom from poisonous substances.

to feeding had by this time been arrested and compensated. The reticulocyte response did not begin until the 8th day but its

TABLE V.

Analyses of Fractions.

Designation of fraction.	Total solids.	Ash.	Total P.	Total N.	Amide and ammonia N.	Protein N.	Non-protein N.
	per cent	*per cent solids*	*per cent solids*	*per cent solids*	*per cent solids*	*per cent solids*	*per cent solids*
II D	6.30	30.00		5.80	1.62		5.80
III D	5.40	29.30	3.20	11.57	2.53	1.40	10.17
IV D	15.72	19.62	2.10	5.56	0.66	0.34	5.22
V D	16.54	21.56	3.08	8.48	1.35	1.82	6.66
VI D	11.86	28.10	3.56	8.44	1.38	1.58	6.86
VII D	19.42	19.10	2.36	9.65	1.22	1.21	8.44
VIII D	14.06	16.74	2.17	7.98	1.27	1.62	6.36
VII G	19.59	20.50	2.97	8.96	1.14	0.90	8.06
VIII G	11.45	20.27	3.50	9.54	1.56	1.04	8.50
IX G	6.94	16.00	3.20	11.60	1.23	2.20	9.40
X G	38.00	20.79	1.70	8.05	1.13	0.66	7.39
XI G	55.60	20.78	2.27	9.51	0.97	0.87	8.64
XII G	37.62	22 33	1.64	8.78	1.09	0.47	8.31
XVII G	Solid.	15.51	3.17	10.05	1.15	0.00	10.05
XIII O	6.58	29.45	0.72	10.84	1.25	0.00	10.84
XIV O	22.03	27.06	1.00	8.51	1.31	0.00	8.51
XV O	39.86	39.05	1.81	8.40	1.00	0.00	8.40

magnitude at the peak was very great. After a fortnight the patient was given raw liver pulp owing to lack of a sufficient supply of extract.[6]

[6] At about this time Eli Lilly and Company of Indianapolis began to manufacture an extract for the Committee on Pernicious Anemia of the Harvard Medical School. As prepared at present this very satisfactory Liver Extract No. 343 is essentially the same as fraction (G), modified however to facilitate manufacture, in part in accordance with a suggestion for which we are indebted to Drs. Best and Scott of the Connaught Laboratories of the University of Toronto. The present extract, though more copious than (G) and richer in carbohydrates, is as satisfactory, therapeutically. Prepared from Liver Extract No. 343, fraction (O) is, however, not free of carbohydrates.

The preparation fed to this patient was free of carbohydrates, as judged by the Molisch test. All three classes of substances, proteins, fats, and carbohydrates, had thus been eliminated without loss of the active principle effective in pernicious anemia. Moreover many organic acids form lead salts and would have passed into fraction (N). There remained chiefly neutral or pre-

TABLE VI.

Analyses of Fractions, Calculated upon Ash-Free Basis.

Designation of fraction.	Total solids.	Total P.	Total N.	Amide and ammonia N.	Protein N.	Non-protein N.
	per cent	*per cent solids*	*per cent solids*	*per cent solids*	*per cent solids*	*per cent solids*
II D	4.41		8.28	2.31	0.00	8.28
III D	3.82	4.52	16.36	3.59	1.98	14.38
IV D	12.64	2.61	6.92	0.83	0.43	6.49
V D	12.98	3.93	10.80	1.72	2.31	8.49
VI D	8.53	4.95	11.73	1.92	2.19	9.54
VII D	15.71	2.91	11.92	1.50	1.49	10.43
VIII D	11.70	2.61	9.59	1.52	1.95	7.64
VII G	15.58	3.73	11.26	1.43	1.13	10.13
VIII G	9.13	4.38	11.95	1.95	1.30	10.65
IX G	5.83	3.81	13.81	1.46	2.62	11.19
X G	30.10	2.14	10.15	1.42	0.83	9.32
XI G	44.05	2.86	12.00	1.23	1.09	10.91
XII G	28.84	2.14	11.45	1.42	0.61	10.84
XVII G	Solid.	3.75	11.89	1.35	0.00	11.89
XIII O	4.64	1.02	15.36	1.76	0.00	15.36
XIV O	16.07	1.37	11.66	1.79	0.00	11.66
XV O	24.30	2.97	13.78	1.64	0.00	13.78

dominantly basic molecules, and in large part, the inorganic constituents of the liver that had passed into fraction (G).

The analyses of the first fifteen preparations are recorded in Table V.[7] If it be assumed that the active principle is not inorganic in nature, these can be recalculated on an ash-free basis. This has been done and the results appear in Table VI. Whereas

[7] These analyses were made by Mr. H. F. Ulrichs and have previously been reported in part (4).

the purer fractions contained less phosphorus, they were richer in nitrogen. The nitrogen content of Preparation XIII O, calculated on an ash-free basis, was 15.36 per cent, of which 1.76 per cent was recovered as ammonia nitrogen. The inference that was tentatively drawn from these results was that the active principle is either a nitrogenous base or a polypeptide.

Phosphotungstate Precipitate (*P*). — Nitrogenous bases and certain polypetides are precipitated by phosphotungstic acid. The former substances are far more soluble than the latter in acetone-water mixtures (1). Sulfuric acid was accordingly added to fraction (O) until it contained 5 per cent by weight, and phosphotungstic acid until precipitation was complete. The precipitate was then repeatedly extracted with 90 per cent acetone, and the acetone-insoluble (R) and soluble (S) phosphotungstates separately regenerated. This was accomplished by adding to each preparation enough 20 per cent barium hydroxide to give a pH of from 8.5 to 9.0 and by filtering off the insoluble barium sulfate and phosphotungstate. Excess barium was then removed by sulfuric acid, or according to a later procedure by a mixture of sulfuric acid and sodium sulfate.

Both the acetone-soluble and insoluble phosphotungstates, (P = R + S), were given to Patient 18. Unfortunately this individual proved to have a very unusual case. She failed to respond by a distinct increased production of reticulocytes after being given this extract, and later did not promptly improve when given whole liver (15). She responded slowly to the feeding of whole liver, however, with an increasing erythrocyte count, which finally reached over 5 million per c.mm.

Another patient was subsequently given this fraction, but it was prepared from a manufactured extract during the period when manufactured extract was not yet satisfactory. None the less the patient responded promptly by an increased, though relatively small number of reticulocytes. This preparation had been further fractionated by precipitating the picrates before the phosphotungstates. After being separately regenerated these fractions were given to the same patient (No. 41). The response in this instance, together with the negative results previously obtained with the phosphotungstate filtrate (Q), suggested that the active principle was precipitable by phosphotungstic acid.

This was made more certain by a communication to us at the time.[8]

Phosphotungstate Filtrate (*Q*).—The filtrate (Q) (separated from the phosphotungstate precipitate fed to Patient 18) was regener-

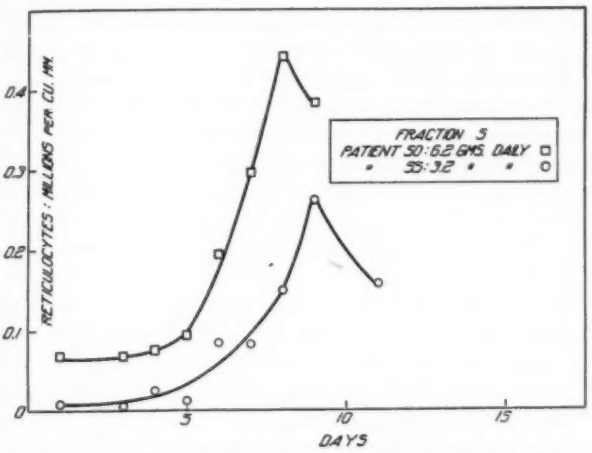


FIG. 6. Change in the reticulocyte concentration, Er, of Patient 50, E_0 = 1.86, and of Patient 55, E_0 = 1.22.

ated and given to another patient (No. 22). The results were entirely negative, suggesting that the active principle had been precipitated by phosphotungstic acid. This patient subsequently responded promptly to the feeding of potent liver extract.

Acetone-Soluble Phosphotungstates (*S*).—Of the substances that

[8] It is a pleasure to acknowledge the cooperation of Dr. Randolph West of the Presbyterian Hospital, New York. Following the announcement of Minot and Murphy, West attempted the fractionation of liver, and independently obtained an extract which contained the active principle (21). The Committee on Pernicious Anemia of the Harvard Medical School has provided him with the extract being manufactured for them by Eli Lilly and Company, so that he might the more easily continue his studies. At about the time that the reticulocytes of Patient 41 were responding weakly to the separately regenerated, but combined phosphotungstates, Dr. West informed us that a case of his had responded by the production of reticulocytes to the unfractionated phosphotungstates (P) made from a lead acetate filtrate (O) which he had prepared following our procedure.

are precipitated by phosphotungstic acid, the phosphotungstates of the nitrogenous bases are more soluble in acetone-water mixtures than are the phosphotungstates of the polypeptides (1). That part of the precipitated phosphotungstates which dissolved readily in 90 per cent acetone-water mixtures has been regenerated separately in our experiments. The solution containing the regenerated acetone-soluble phosphotungstates derived from 27 kilos of liver contained 55.9 gm. of solids. Of this Preparation XXXV S, 6.2 gm. were given daily to Patient 50. The reticulocyte response was so great as to indicate that this fraction was a concentrated source of the active principle (Fig. 6).

In studying Preparation XXXV S it was noted that a precipitate appeared in acid solution. Accordingly another preparation, No. XL S, was made and the acid-precipitable material removed from it before it was given to a patient. The extract from 120 kilos of liver was employed in this preparation and yielded 191.2 gm. of regenerated acetone-soluble phosphotungstates, from which the acid-precipitable material had been removed. The extract derived from 2 kilos of liver containing 3.2 gm. of solids was given to Patient 55 daily. The reticulocyte response clearly indicated that the acetone-soluble phosphotungstates contained the active principle.

Acetone-Insoluble Phosphotungstates (R).—After regenerating the acetone-insoluble phosphotungstates (R) it was noticed that a precipitate appeared at acid reactions. This precipitate appeared to reach its maximum in the neighborhood of pH 1.5 or 2, where it was readily flocculated. It was much more soluble at high than at low temperatures, and more soluble in salt solution than in distilled water. In Preparation XXXV, fraction (R) was concentrated and brought to pH 2 at a low temperature. The resulting precipitate was removed by centrifugation and filtration, washed with 0.1 N acetic acid, and redissolved by means of sodium hydroxide to a neutral reaction. The yield from 54 kilos of liver was 6.6 gm.

The acid-precipitable material appeared to be similar in fractions (R) and (S). The conditions affecting the separation and the regeneration of Preparation XL were therefore altered as we have seen so that the acid-precipitable material would pass largely into fraction (R). Hydrochloric acid was used as the precipitant

and as the wash water of the acid-precipitable material, which was dried by means of alcohol and ether. The yield of this acid-precipitable material from 120 kilos of liver was 59 gm. in this instance.

1.2 gm. of the acid-precipitable material in fraction (R) of Preparation XXXV, were given daily to Patient 53 for 5 days. There followed a slight reticulocyte response. On the 6th day no extract was available, but starting on the 7th day 2 gm. of the same fraction of Preparation XL, were given daily for 5 days. An additional reticulocyte response followed (Fig. 7). The patient thus received 16 gm. of fraction (R) in 11 days, or approximately 1.5 gm. per day. The experiment indicated that the amount fed per

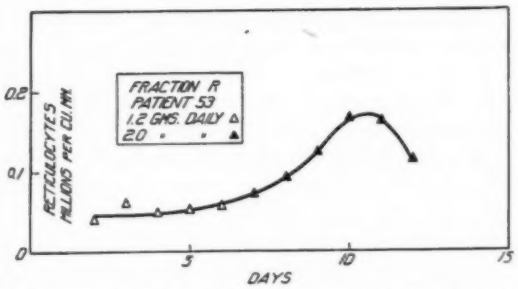


FIG. 7. Change in the reticulocyte concentration, Er, of Patient 53, $E_0 = 1.01$.

day was less than enough to produce the maximum reticulocyte response, but that this fraction contained the active principle.

Although fraction (O) had been high in nitrogen, the acid-precipitable material in fraction (R) contained but 7.11 per cent of nitrogen. Moreover it contained 1.10 per cent of phosphorus and 62 per cent ash. Since no acid-precipitable material could be observed in (O), it appeared probable that a small amount of phosphotungstic acid had not been removed as a barium salt at the alkalinity and in the volumes employed in regenerating these preparations. This hypothesis was rendered the more probable since the ratio of phosphorus to ash suggested the ratio of phosphorus to tungstic oxide in phosphotungstic acid. The conditions which prevented the complete precipitation of the phos-

photungstic acid as a barium salt are being investigated, as well as the precise conditions for the precipitation of the active principle by phosphotungstic acid.

Calculated on an ash-free basis, the nitrogen content of the acid precipitate in Preparation XL, fraction (R), was 18.7 per cent. This precipitate may thus be considered as consisting of phosphotungstates of nitrogenous substances. On this basis but 0.6 gm. constituted the average amount of nitrogenous substances given daily to Patient 53.

Of the regenerated phosphotungstates in Preparation XL, fraction (R), 94 per cent are precipitable by silver, and 92 per cent by mercuric acetate at an alkaline reaction. With a product as pure as fraction (R) it is hoped that the metal precipitations which have been repeatedly attempted upon less pure fractions may be more successfully employed. Earlier results are not reported in detail because of the losses in activity heretofore suffered in effecting these separations and regenerations. It may be recorded, however, that the regenerated silver precipitate (V) fed to a patient (No. 26) appeared inert, whereas the filtrate (W) produced a weak though definite reticulocyte response in Patient 23. On the other hand the mercury filtrate (Y) fed to Patients 34 and 51 was inactive, as was the material precipitated by mercuric sulfate from solution in sulfuric acid, whereas the material precipitated by mercuric acetate (X) from neutral solution has been shown to contain the active principle (Patient 65).

Like certain of the previous preparations, Preparation XL (R) was free of carbohydrates. A 1 per cent solution gave no Molisch test, no test with Tollens' reagent, and did not reduce Benedict's solution. It also failed to give the lead-blackening test characteristic of reduced sulfur. It gave a strong biuret,[9] a strong Millon's, but only a slight xanthoproteic reaction. It gave a strong reaction with diazobenzene sulfonate and with Folin's phenol reagent, but not with his uric acid reagent. It gave a faint Adamkiewicz reaction and a positive test with dimethylaminobenzaldehyde. The α-naphthol-hypochlorite and the diacetyl tests were also positive. The absence of certain substances is thus demonstrated, but no final deductions will be drawn at this time regarding the

[9] Although Dr. West reported (21) that his extract gave no biuret test, he has informed us that his subsequent fractions have all given strong biuret reactions.

nature of the substances present in the purest fractions that have thus far been effective in pernicious anemia.

Effect of the Active Principle upon Immature Red Blood Cells.

Throughout this investigation the effort has been made to find some method of testing for the active principle effective in pernicious anemia that did not involve feeding patients fractions of liver. During the period when these fractions were being studied Dr. Sabin added certain of our extracts to chick embryo preparations and observed what seemed to be an accelerated division rate of certain cells.[10] She reports: "In a series of 266 early blastoderms mounted in Locke solution, to which had been added chicken bouillon as prepared by Lewis and Lewis, no second division of the cells of a given blood island was observed in intervals of from 3 to 6 hours" (19). "In three successive preparations in which extract [Preparation XL R] had been added to the medium a second cell division was seen. In two preparations the second division was of a blood island and the interval approximated three hours; in the third preparation the second division was of an endothelial cell, making the wall of the vessel, and the interval was approximately one hour. The preparations were fixed just as the second cell division was observed and they confirm the observation that the blood islands in the two preparations and the endothelium in the third were in a cycle of cell division." "In all of these chicks the heart action remained very vigorous throughout the experiment, so that there was no sign of any toxic substance involved." "This material is interesting inasmuch as the blood islands during the stages studied are made up entirely of the megaloblast." Megaloblasts and similar primitive cells crowd the bone marrow in pernicious anemia in relapse. Peabody concluded (18) from direct observations upon the bone marrow of pernicious anemia that liver feeding reduced the proportion of megaloblasts in this tissue. The appearance in the peripheral blood of reticulated red blood cells following the treatment of this disease by means of the active principle in liver has been interpreted as due to the maturation of these primitive cells (15).

[10] It is a pleasure to acknowledge the collaboration of Dr. Florence R. Sabin. We are indebted to her for the above report upon her experiments with Preparation LX, fraction (R).

These observations suggested that the active principle effective in pernicious anemia may have an effect in accelerating the development of primitive red blood cells. With this in mind small amounts of the purest fractions that had been prepared and were known to be effective in pernicious anemia were added to a suspension containing reticulated red blood cells *in vitro*. Histological observations were made by one of us (Minot) upon the staining of the reticulated cells by means of brilliant cresyl blue. Observations upon metabolism were made for us by Dr. Robert Emerson. The results that have been obtained thus far, though inconclusive, suggest that the purest fractions thus far prepared increase the metabolism and influence the staining of immature red blood cells either by reducing the dye or by modifying the reticular material.[11] Whether or not these phenomena depend upon impurities in even these preparations or upon the active principle effective in pernicious anemia must await further experimentation.

The purest fractions of the liver thus far prepared that contain the active principle effective in pernicious anemia have now been observed to accelerate one of the first stages in the formation of red blood corpuscles, namely the division rate of primitive cells, and may modify one of the last stages. These observations have so modified the course of this research as to render it desirable at this time to report so much of the attempt to isolate the active principle as has completely depended upon the feeding of fractions of liver to patients with pernicious anemia. This part of the investigation suggests that the active principle is a nitrogenous base or polypeptide.

Further studies of the nature of the active principle will depend in large part upon observations of the action of this, or of a related substance, upon the modification of different types of cells. The

[11] Liver Extract No. 343 has been shown by Dr. West to reduce the dye under anaerobic conditions (personal communication). Our purest fractions exhibit but a very slight reducing action on brilliant cresyl blue however, or on methylene blue even in the presence of such substances as glucose, dihydroxyacetone, formaldehyde, or xanthine. The notion that we might be concerned with an oxidizing enzyme led to testing Preparation XL, fraction (R) by means of guiaconic acid, phenolphthalin, and pyrogallol in the presence of hydrogen peroxide. No peroxidase action was observed.

relation between the active principle effective in pernicious anemia and those substances extracted from liver by Heaton (7), that influence the growth of fibroblasts, must be considered. These considerations will involve studies of the metabolism and the kinetics of the maturation process. Thus we may pass from the study of a special problem of clinical medicine to one of general biology.

SUMMARY.

1. Studies are reported of the nature of the active principle effective in pernicious anemia. In these studies fractions of liver were fed to patients with this disease. Those fractions were considered to contain the active principle that produced an increased concentration of reticulocytes and erythrocytes in the peripheral blood.

2. The relations between the reticulocytes and the erythrocytes in the blood during the first days of feeding a potent extract have been mathematically formulated.

3. The active principle is soluble in water, insoluble in ether, and precipitable by alcohol.

4. Fractions have been prepared free of iron and of proteins, carbohydrates, and lipoids and have proved potent sources of the active principle. The active principle is found in the filtrate from basic lead acetate and is precipitable by phosphotungstic acid.

5. A fraction of the phosphotungstates contains the active principle. This consists of phosphotungstates of substances containing about 19 per cent of nitrogen. The active principle must therefore be considered either a nitrogenous base or a polypeptide of which but 0.6 gm. a day has sufficed to produce a pronounced response of reticulated red blood corpuscles in a patient with pernicious anemia.

6. The growth of immature red blood corpuscles has been stimulated by the purest fractions thus far prepared containing the active principle effective in pernicious anemia. The special methods and problems of clinical medicine are therefore being supplemented by those of general biology in the study of this constituent of liver.

XUM

BIBLIOGRAPHY.

1. Barger, G., The simpler natural bases, London and New York, 1914.
2. Best, C. H., Dale, H. H., Dudley, H. W., and Thorpe, W. V., *J. Physiol.*, 1927, lxii, 397.
3. Bradley, H. C., *J, Biol. Chem.*, 1922, lii, 467.
4. Cohn, E. J., Minot, G. R., Fulton, J. F., Ulrichs, H. F., Sargent, F. C., Weare, J. H., and Murphy, W. P., *J. Biol. Chem.*, 1927, lxxiv, p. lxix.
5. Dakin, H. D., *Biochem. J.*, 1918, xii, 290.
6. Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 297.
7. Heaton, T. B., *J. Path. and Bact.*, 1926, xxix, 293.
8. James, A. A., Laughton, N. B., and Macallum, A. B., *J. Am. Med. Assn.*, 1926, lxxxvii, 317.
9. Levene, P. A., and van der Hoeven, B. J. C., *J. Biol. Chem.*, 1924, lxi, 429.
10. Major, R. H., *J. Am. Med. Assn.*, 1926, lxxxvii, 311.
11. Minot, G. R., Cohn, E. J., Murphy, W. P., and Lawson, H. A., *Am. J. Med. Sc.*, 1928, clxxv, 599.
12. Minot, G. R., and Murphy, W. P., *J. Am. Med. Assn.*, 1926, lxxxvii, 470.
13. Minot, G. R., and Murphy, W. P., *J. Am. Med. Assn.*, 1927, lxxxix, 759.
14. Minot, G. R., Murphy, W. P., Cohn, E. J., Stetson, R. P., and Lawson, H. A., *Tr. Assn. Am. Phys.*, 1927, xlii, 83.
15. Minot, G. R., Murphy, W. P., and Stetson, R. P., *Am. J. Med. Sc.*, 1928, clxxv, 581.
16. Murphy, W. P., and Blotner, H., *J. Clin. Inv.*, 1927, iv, 440.
17. Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.
18. Peabody, F. W., *Am. J. Path.*, 1927, iii, 179.
19. Sabin, F. R. *Carnegie Institution of Washington, Pub. No. 272, Contributions to Embryology*, 1920, ix, 213.
20. Voegtlin, C., Neill, M. N., and Hunter, A., *Bull. Hyg. Lab., U. S. P. H. S., No. 116*, 1920.
21. West, R., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 665.

STUDIES ON GLYOXALS.

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I. A Colorimetric Method for Glyoxals.

The observation that the addition of alkali cyanide to a solution of glyoxal or methylglyoxal causes in alkaline solution an immediate great increase of the reducing intensity—as indicated for example by instant reduction of methylene blue or indigo carmine —suggested the utilization of this behavior for the quantitative determination of glyoxals. After trials of various oxidizing agents, the arsenophosphotungstic acid reagent used by Benedict (1) for uric acid seemed to be suitable and the optimum conditions were sought to secure maximum color, stability, and proportionality. The rather notable effect of cyanide in greatly increasing the reducing power and intensity is not limited to glyoxals, though it is exceptionally striking with these substances. The action of cyanides in the uric acid determination, as introduced by Benedict, and its effect in causing cystine and denatured protein to give reactions with nitroprusside (2), have probably a similar explanation. The mechanism of the action is presumably the formation of an unstable addition product like cyanhydrin in character, though we have not succeeded so far in securing direct evidence that this is the case. The conditions chosen for the determination of glyoxals are similar to those in Benedict's uric acid method, and uric acid and possibly other substances will therefore interfere, if present, in the solution being analyzed.

1. Reagents.

1. Benedict's Uric Acid Reagent.—100 gm. of pure sodium tungstate are dissolved in a liter flask in about 600 cc. of water. 50 gm. of pure arsenic pentoxide, 25 cc. of 85 per cent phosphoric

acid, and 20 cc. of concentrated hydrochloric acid are added in order. The mixture is boiled gently for 20 minutes, cooled, and diluted to the mark. The greenish blue color which may develop can be decolorized by boiling with a drop of bromine.

2. 1 M Sodium Cyanide Solution.

3. 1 M Sodium Carbonate Solution.

4. Standard Glyoxal and Methyl Glyoxal Solutions.

Glyoxal was prepared by de Forcrand's method (3) of oxidizing paraldehyde by nitric acid and distilling the resulting mixture *in vacuo* (purity 94 per cent). Methylglyoxal was prepared from dihydroxyacetone according to the method of Fischer and Taube (4). Both solutions are standardized by the methods described by Friedemann (5). 10 cc. of 0.001 M solution are taken as the standard, since this amount gives a suitable depth of color with the reagents.

2. Conditions for Color Development.

The more important factors proved to be the alkalinity and concentrations of arsenophosphotungstic acid, of cyanide, and of glyoxals. With NaOH the color developed was inconstant and low, due perhaps to too rapid destruction of the glyoxals. Alkali carbonate appears to provide suitable alkalinity. With 10 cc. of 0.001 M glyoxal and 2 cc. of arsenophosphotungstic acid reagent, 5 cc. of M Na_2CO_3 and 1 cc. of M KCN proved to be optimum quantities and were adopted as the standard procedure. With larger amounts of carbonate a somewhat deeper color was obtained after 10 minutes, but the results were less constant. Less cyanide gave slightly lower results, while 2 cc. caused a precipitation or cloudiness. When the amounts stated are mixed, allowed to stand 10 minutes at room temperature, and diluted to 100 cc., the solution remains clear and the color unchanged for at least 2 hours. If the mixture is diluted after 5 minutes the color is about 2 per cent less, while if dilution is postponed to 15 minutes or longer a precipitation occurs. In order to secure constant and comparable results it is therefore essential to adhere to certain conditions as to amounts of reagents, volume of solution, and time of reaction. If the total volume of the reaction mixture is less than that stated (18 to 20 cc. for the amounts of reagents given) precipitation occurs; if larger, the rate of color development

is slower and the results low. Heating the mixture during the reaction yields low results.

The behavior of methylglyoxal toward the reagents is the same

TABLE I.

Determination of Varying Amounts of Glyoxal and Methylglyoxal.

10 cc. of glyoxal solution + 2 cc. of arsenophosphotungstic acid reagent + 1 cc. of M NaCN + 5 cc. of M Na_2CO_3. After 10 minutes at room temperature (about 25°), the mixture was diluted to 100 cc. (or to other volume as stated).

Amounts of glyoxal in cc. of 0.001 M.	Colorimeter readings.		Found.			
			Glyoxal, in per cent of:		Methylglyoxal, in per cent of:	
Total volume of reaction mixture kept constant at 18 cc.	Glyoxal.	Methyl-glyoxal.	Standard.	Amount present.	Standard.	Amount present.
	mm.	*mm.*				
8	25.3		79	99		
9	22.8		88	98		
9.5	22.0		91	96		
10.0 Standard.	20.0	20.0				
15.0	15.0	15.0	133	89	133	89
20.0	26.8*	25.8*	149	75	155	77
30.0	32.2†		186	62		
With half quantities of reagents as above stated. Total volume of reaction mixture 9 cc.; diluted after 10 minutes to 50 cc. Same standard as above.						
5	19.9	20.0	50	100	50	100
3	31.9	33.6	31	103	30	100
2	26.8‡	26.9‡	75	93	74	93
1	37§	38§	81	81	79	79
0.5	Too faint.					

* Diluted 1:1 before reading.

† Diluted 1:2 before reading.

‡ Standard diluted 1:1.

§ Standard diluted 1:4 and set at 30.

as that of glyoxal except that the depth of color is only 74 per cent of the latter. With methylglyoxal the amount of the reagents should be reduced to 80 per cent of that used with glyoxal.

There are limitations also as to the amount of glyoxals which may be used without loss of proportionality in color produced, as shown in Table I.

The minimum amount of glyoxals determinable under the conditions used is 1 cc. of 0.001 M solution.

3. Influence of Nature of Buffer on the Determination of Glyoxals.

When glyoxals are determined in a buffer solution, attention must be paid to the following points. Phosphate buffer causes heavy precipitation with the reagents. In cases where the use

TABLE II.

Color Development of Various Substances.

Concentration = 0.0001 M per liter.

	Color development.
Glucose	±
Fructose	±
Dihydroxyacetone	= 20 per cent methylglyoxal.
Formaldehyde	−
Acetaldehyde	++
Glyceric aldehyde	+
Glycolaldehyde	+++
Lactic acid	±
Pyruvic acid	−
Creatine	−

of phosphate is unavoidable, however, the solution may be centrifuged and the supernatant liquid used without introducing serious error. When 0.1 M citrate-0.1 N NaOH or 0.05 M borate-0.2 M boric acid mixture is used, the amount of Na_2CO_3 must be increased to 6 cc. to get the optimum alkalinity for the color development.

4. Removal of Protein from the Fluid Being Tested.

Among many reagents trichloroacetic acid seemed most suitable for the removal of protein from the solutions to be analyzed. The use of amounts up to 1 cc. of 10 per cent trichloroacetic acid per 10 cc. of solution may be allowed for the determination without any ill effect.

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5. Influence of Presence of Various Substances on Color Development.

The following substances were examined for their influence on the determination: glucose, fructose, dihydroxyacetone, formaldehyde, acetaldehyde, glyceric aldehyde, glycolaldehyde, lactic acid, pyruvic acid, creatine, and creatinine. Some of these substances reacted with the reagents but the color was not deep enough to be read in the colorimeter tube except in the case of dihydroxyacetone which gave about 20 per cent of the color of methylglyoxal of the same concentration (Table II). This color development of dihydroxyacetone may possibly be due to the presence of methylglyoxal in preparations of dihydroxyacetone. In our method, 0.86 mg. of uric acid gives the same depth of color as 0.72 mg. (10 cc. 0.001 M) of methylglyoxal. This amount of uric acid may be used as the standard, although the tint of color is not quite the same.

6. Determination.

A. Glyoxal.—To 10 cc. of glyoxal solution to be tested are added, in order, 2 cc. of arsenophosphotungstic acid reagent followed by 1 cc. of M NaCN and 5 cc. of M Na_2CO_3. After standing 10 minutes the mixture is diluted to 100 cc. with water and the depth of the color is read against a standard in which 10 cc. of 0.001 M glyoxal solution and the same amount of the reagents as mentioned above are treated in the same way. With this amount of the reagents we can determine within about 5 per cent from 0.58 to 0.87 mg. (= 10 to 15 cc. of 0.001 M solution) of glyoxal. With smaller amounts of glyoxal a precipitate forms unless smaller amounts of reagents are used. With one-half the usual amounts of reagents quantities of glyoxal down to 0.06 mg. may be determined. Beyond this minimum the color has a slight red nuance which makes its comparison with the diluted standard impossible. When a proper amount of glyoxal (0.6 to 0.15 mg.) is chosen, the error does not exceed about 5 per cent. Unless the concentration of glyoxal in the solution to be analyzed is approximately known, it is necessary to make a preliminary determination for orientation. Another portion of the solution is then diluted, or the standard glyoxal solution is diluted, so that approximately equal amounts in unknown and standard may be taken for the determination.

B. Methylglyoxal.—To 10 cc. of methylglyoxal solution to be tested are added 1.6 cc. of arsenophosphotungstic acid, 0.8 cc. of M

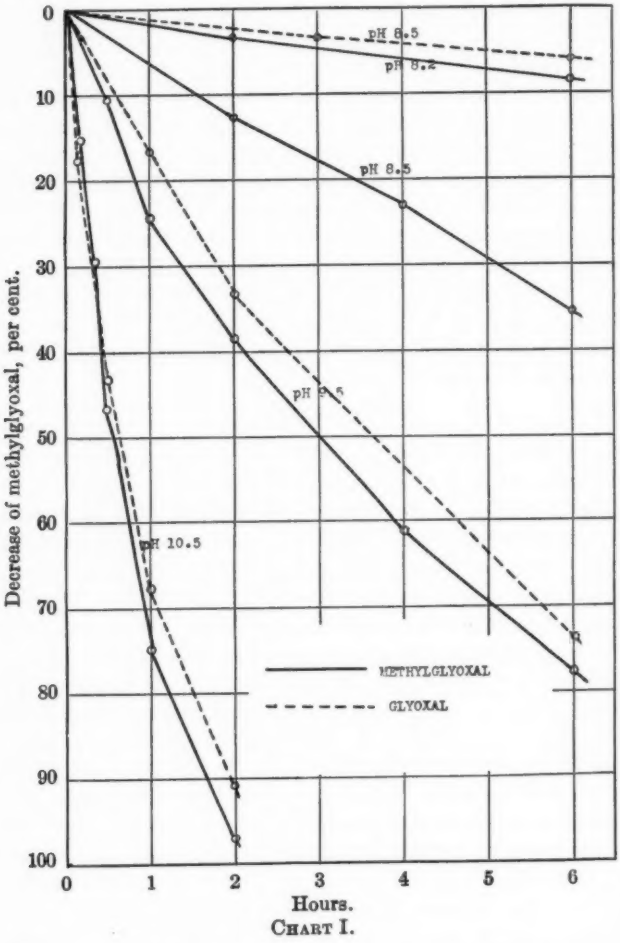

CHART I.

NaCN, and 4 cc. of M Na_2CO_3 in succession. The mixture is treated in the same way as glyoxal. We take 10 cc. of 0.001 M methylglyoxal as the standard.

II. Influence of Hydrogen Ion Concentration on Glyoxal and Methylglyoxal.

It is well known that glyoxals although stable in acid solution rapidly change to corresponding hydroxy acids in the presence of alkalies. Shaffer and Friedemann (6) observed that glyoxal was converted very rapidly to glycollic acid at pH 12.5 at 25°, more slowly at pH 11, and was stable at pH 7.5. It was shown by Friedemann (5) that the same relations appear to hold also for methylglyoxal. It was desired to obtain more information as to the conditions under which this transformation occurs by following its rate by means of the colorimetric method already described. 10 cc. of 0.01 M glyoxal or methylglyoxal solution were diluted to 100 cc. with buffer solutions of varying hydrogen ion concentrations and placed in the thermostat at 37.5°. To determine the remaining glyoxal, 10 cc. of this fluid were pipetted out at intervals, added to the reagents, and read in the colorimeter against the standard. As the buffer, Palitzsch's borate-boric acid mixture was used in the experiments at pH 8.2 and $NaHCO_3$ (0.5 M)-Na_2CO_3(0.5 M) mixture in the other experiments. pH of the buffers was determined electrometrically. The results are shown in Chart I.

At pH 8.0 glyoxal was quite stable while methylglyoxal showed 2.6 per cent decrease after 6 hours.

When the results at pH 8.5, 9.5, and 10.5 are plotted they fall on fairly regular curves which conform to a monomolecular reaction. The calculated monomolecular velocity constants are moderately satisfactory considering the limited accuracy of the analytical method. Taking what appear to be representative values of K (log e) at each pH we have the relations shown below.

pH	$K \times 10^4$ (log e)	pK (= − log K)	pH × pK
8.5	2.4	3.62	30.8
9.5	32.2	2.49	23.7
10.5	177.3	1.75	18.4

Although the results can be regarded only as approximate, they seem to show that, as might be expected, the transformation of glyoxal at a given pH proceeds at the rate of a monomolecular

reaction and that the rate increases slightly more slowly than the hydroxyl ion concentration of the solution.

With methylglyoxal the same relations appear to hold.

pH	$K \times 10^3$ (log e)	pK	pH × pK
8.5	1.38	2.86	24.3
9.5	3.69	2.43	23.1
10.5	18.42	1.73	18.2

Formation of Lactic Acid from Methylglyoxal.

With the expectation that the decrease of methylglyoxal would be paralleled by the formation of lactic acid the latter substance was determined by the Friedemann, Cotonio, and Shaffer method

TABLE III.

Formation of Lactic Acid from Methylglyoxal at pH 9.0 and 10.5.

5 cc. of 0.1 M methylglyoxal were diluted to 100 cc. with carbonate-bicarbonate buffer at 37.5°.

Time.	pH 9.0.		pH 10.5.	
	Decrease of methylglyoxal.	Increase of lactic acid.	Decrease of methylglyoxal.	Increase of lactic acid.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*
0.5			47.1	5.0
1.0	6.7	2.6	74.6	7.0
1.5			81.3	11.0
3.0	25.5	3.0		
5.0	31.0	5.0		

(7), as formed from methylglyoxal, at pH 9.0 and 10.5. The results, given in Table III, are surprising in that the appearance of lactic acid falls far behind the amount of methylglyoxal which disappeared.

It was proved that lactic acid formed from methylglyoxal did not suffer any serious further change in the conditions of the experiment. That the presence of methylglyoxal up to a certain amount has no serious influence on the determination of lactic acid is shown in Table IV.

The results show that the presence of 3 cc. of 0.04 M methyl

glyoxal (= 8.64 mg.) has no serious influence on the lactic acid determination; larger amounts give high results.

The data of Table III seem to hint at the formation of an intermediary substance in the reaction which involves the lactic acid formation from methylglyoxal. At lower hydrogen ion concentration than pH 12 the alkalinity might not be strong

TABLE IV.

Influence of Methylglyoxal on Lactic Acid Determination.

10 cc. of M/300 zinc lactate (6.0 mg. of lactic acid) + 0.04 M methylglyoxal.

	Zinc lactate alone.	Amount of 0.04 M methylglyoxal.					
		1 cc.	2 cc.	3 cc.	4 cc.	5 cc.	10 cc.
Lactic acid recovered, *mg.*	5.82	5.93	5.99	6.09	6.82	7.09	8.12
" " in methylglyoxal (control), *mg.*		0.11	0.16	0.18	0.55	0.73	1.50
Lactic acid recovered, *per cent.*	97.0	97.0	97.1	98.5	104.5	106.0	110.4

TABLE V.

Lactic Acid Formation from Supposed Intermediate.

Time.	Methylglyoxal.		Lactic acid.		
	Decrease.	Remaining.	Formed at pH 10.5.	After excess alkali.	Formed by alkali.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.5	47.8	52.2	5.0	51.0	46.0
1	64.5	35.5	6.0	41.3	35.3
2	80.0	20.0	10.0	34.3	24.3
4	93.5	6.5	13.0	24.5	11.5
52	100.0	0	17.5	20.2	2.7

enough to convert methylglyoxal so far as lactic acid, a large part of methylglyoxal possibly remaining at an intermediary stage. If this be the case, lactic acid must be easily formed from the intermediate by increasing the alkalinities to pH 12 or so. Based upon this expectation the following experiment was carried out.

10 cc. of 0.1 M methylglyoxal were made up to 200 cc. with carbonate-bicarbonate buffer solution (pH 10.5) and kept at 37.5°.

At suitable intervals small parts (10 cc.) were boiled gently with an excess of alkali (10 cc. of N NaOH) to convert the remaining methylglyoxal and hypothetical intermediate to lactic acid.

The results in Table V show that the warming with excess alkali converted the remaining methylglyoxal into lactic acid (last column, Table V) but that no additional lactic acid was formed from that part of the methylglyoxal which had already been destroyed at the buffer pH. In other words the product resulting from the transformation of methylglyoxal does not give rise (with excess alkali) to lactic acid and therefore cannot be regarded as an intermediate in the methylglyoxal → lactic acid reaction.

Assuming that the failure of the lactic acid production from some intermediary substance might be due to oxidation of the

TABLE VI.

Action of Alkali on Methylglyoxal.

15 cc. of 0.05 M methylglyoxal and 50 cc. of 0.1 N NaOH in 150 cc. at about 25°. The increase of acidity was calculated with the assumption that 1 molecule of a monobasic acid was formed from 1 molecule of methylglyoxal.

Time.	Decrease of methylglyoxal.	Increase of lactic acid.	Increase of acidity.
min.	*per cent*	*per cent*	*per cent*
5	51.7	50.5	55.17
10	73.6	63.2	68.96
20	92.6	82.5	87.9
40	100±	87.5	100±

substance by the air during the experiment, the methylglyoxal solution in pH 10.5 buffer was aerated with the air at 37.5° and the amount of lactic acid formed was compared with that of the solution which was covered with mineral oil. A noticeable difference in lactic acid production was not observed.

The supposition, that such a reactive substance as methylglyoxal might form a stable condensation product in weakly alkaline reaction, is perhaps more reasonable than that some chemical change might take place in the molecule of methylglyoxal to form an unknown derivative not convertible into lactic acid.

The possibility suggested itself that a synthesis of triose or hexose phosphate from methylglyoxal and phosphate might

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occur at low hydrogen ion concentration, but that this was not the case was proved from the fact that no decrease of free phosphate was observed in the mixture of methylglyoxal and disodium hydrogen phosphate at pH 10.5. Thus, while the fate of methylglyoxal at lower hydrogen ion concentration is unknown, the figures in Table VI show clearly that at higher alkalinity (pH about 12.5) the decrease of methylglyoxal kept pace with the increase of lactic acid and acidity, indicating almost a quantitative conversion of methylglyoxal to lactic acid. This confirms the results of Friedemann (7).

III. Action of "Glyoxalase" on Glyoxal and Methylglyoxal.

According to Dakin and Dudley (8) there is, in practically all animal tissues except pancreas, an enzyme which converts glyoxals to corresponding hydroxy acids. They proposed that it be named "glyoxalase." At the same time Neuberg (9) found the same enzyme in dog liver and muscle and named it "ketonaldehydmutase." Foster (10) repeated Dakin and Dudley's experiment and got similar results with the exception that rabbit muscle appeared not to contain glyoxalase. Dudley (11) showed the last observation to be erroneous by detecting the presence of the enzyme in weak alkaline (Na_2CO_3) extract of rabbit muscle. He attributed Foster's observation to inhibition of the action of the enzyme by acid produced in the muscle.

Glyoxalase which was used in the present experiments was prepared by Dakin and Dudley's method. To 1 part of finely minced fresh animal tissues were added 5 parts of distilled water. After an hour's standing at room temperature with occasional stirring the mixture was filtered through paper. This turbid extract was used as the source of the enzyme. The filtrates were always kept in the cold room at 0–7° until used. As the glyoxalase content was richest in liver the author has used mainly rabbit liver extract.

Animal tissues contain some substance which gives the blue color with Benedict's reagent, in an amount corresponding to 3 to 4 mg. in terms of methylglyoxal per 100 gm. of tissue. Bearing in mind that uric acid gives the reaction, we tried the following experiment to determine the nature of the substance. Fresh liver and muscle hash were extracted for an hour in the cold room with water which had been acidified to prevent the destructive

action of glyoxalase on methylglyoxal, if present. The extracts were treated with Morris and Macleod's reagent (12) ($ZnCl_2$ and

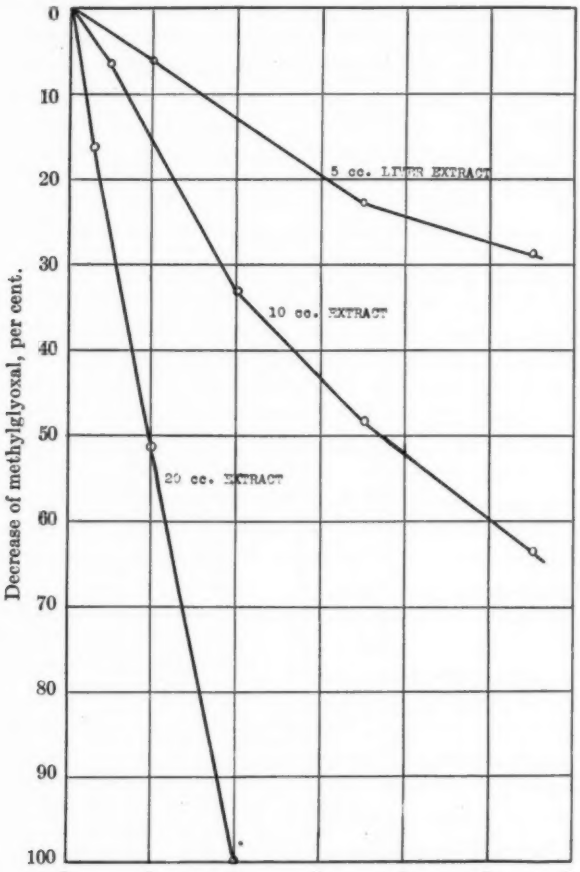

CHART II.

Na_2CO_3) to remove uric acid from the extract. The filtrate from the $ZnCO_3$ precipitate did not give the blue color. From the

precipitate dissolved in HCl almost the whole substance was recovered, showing the complete removal of the substance with $ZnCO_3$. Thus the blue color developed in tissue extracts is

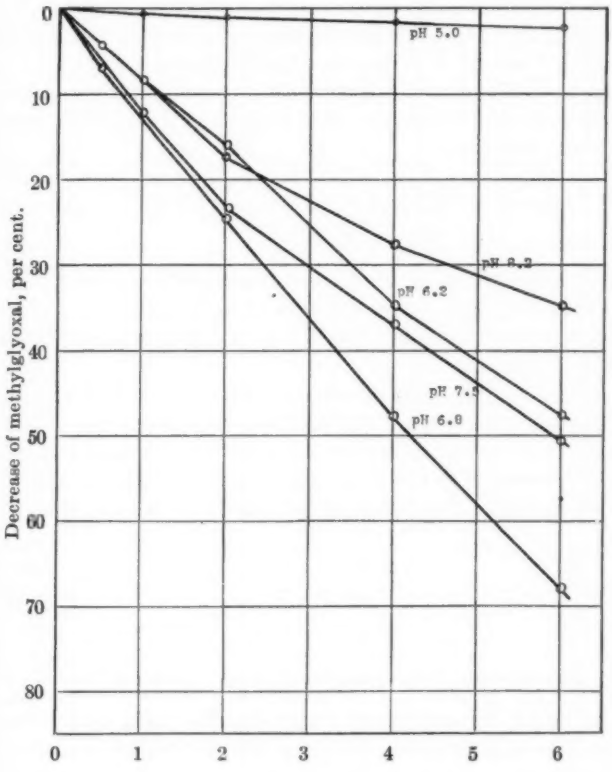

CHART III.

apparently due to the uric acid (or other purines) present in the tissues. Methylglyoxal, a probable intermediate of carbohydrate metabolism, is not present in detectable quantities in such tissue extracts.

1. Action of Glyoxalase on Methylglyoxal.

To 4 cc. of 0.1 M methylglyoxal were added 20, 10, and 5 cc. of rabbit liver extract (48 hours old). The mixtures were diluted to 100 cc. with borate-boric acid mixture of pH about 7.5 and incubated at 37.5°. At intervals, 10 cc. of each fluid were pipetted out, added to 4 cc. of 10 per cent trichloroacetic acid and 6 cc. of water, and shaken well. After standing 10 minutes the fluid was filtered and 5 cc. of the filtrate were examined for the methylglyoxal content (Chart II).

2. Influence of Hydrogen Ion Concentration upon Glyoxalase Activity.

A. Methylglyoxal.—4 cc. of 0.1 M methylglyoxal + 10 cc. of liver extract (2 days old) + buffer at 37.5° to 100 cc.

Buffer.—Citrate-NaOH at pH 5.0, 6.2, 6.8; borate-boric acid at pH 7.5, 8.2.

A glance at Chart III shows that the optimum hydrogen ion concentration for the activity of glyoxalase lies in the neighborhood of pH 7.0. The values of velocity constants calculated from the reactions in the chart approximately satisfy a monomolecular reaction. The results of calculation are shown below.

pH	$K \times 10^4$	pK	pH × pK
6.2	15.0	2.82	17.5
6.8	24.2	2.62	17.8
7.5	19.6	2.71	20.2

The buffer capacity of the borate solution used in the experiments was so low that the change in pH resulting from lactic acid formation was quite large; the pH values given represent only the initial condition.

Meyerhof (13) has observed a lower yield of lactic acid from old methylglyoxal samples which he ascribed to a slow decomposition of the glyoxal into some unknown substance in the course of time. As suggested by this observation we have compared the action of glyoxalase on a fresh solution prepared by dissolving fresh greenish vaporous methylglyoxal in water with the action of

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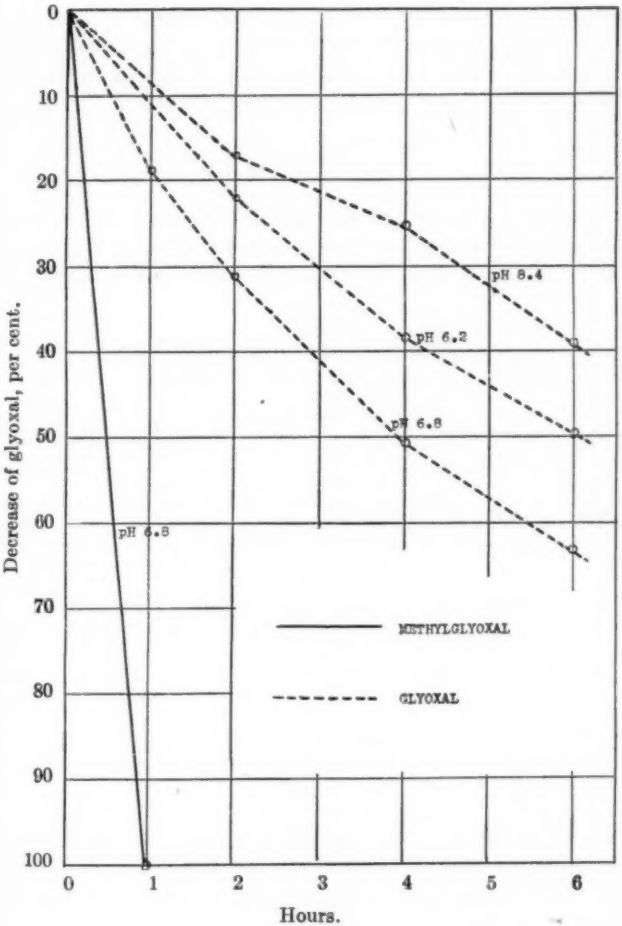

CHART IV.

the same enzyme on a similar solution some days old. There was no difference in the rates of conversion.

B. Action of Glyoxalase on Glyoxal.—Similar experiments were performed with glyoxal under the same conditions as methylglyoxal with the exception of using 20 hours old liver extract.

As shown in Chart IV it is striking that glyoxal is much more resistant to the action of the enzyme than is methylglyoxal. After an hour's incubation only 18.91 per cent of glyoxal disappeared at pH 6.8, while under the same conditions all of the methylglyoxal (control) was destroyed.

3. Influence of Time on Activity of Glyoxalase.

During these experiments it was often observed that a rather rapid weakening of the activity of glyoxalase occurred in the course of time. The following experiment is in conflict with Dakin and

TABLE VII.

Influence of Time on Activity of Glyoxalase.

0.0208 gm. of methylglyoxal + 2 gm. of liver (extract).

Time of incubation with methylglyoxal.	Decrease of methylglyoxal.						
	pH 6.2.				pH 6.8.		
	Time of incubation of liver alone.						
	At start (fresh).	After 18 hrs.	After 24 hrs.	After 48 hrs.	At start (fresh).	After 42 hrs.	After 66 hrs.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.5	100	11.1		4.4	100±	5.7	
1		25.1	13.0	8.4		13.7	12.0
2		47.0	26.5	16.0		32.3	24.4
4		92.5	56.0	34.9		69.2	47.9
6		100±		47.4		100±	68.0

The experiments at pH 6.2 indicate about 40 per cent diminution of the activity every 24 hours and the experiments at pH 6.8 show about 30 per cent weakening.

Dudley's description: "On allowing the tissue extract to stand at room temperature, without addition of antiseptics, the activity is lost relatively slowly. After forty-eight hours there is not much diminution in activity . . . . " (8). From the liver extract (kept in the cold room during the experiment) 10 cc. were pipetted out at intervals, added to 4 cc. of 0.1 M methylglyoxal, and diluted to 100 cc. with citrate-NaOH buffer (pH 6.8 and 6.2). The mixtures were incubated at 37.5° and at intervals examined for methylglyoxal content (Table VII).

4. Influence of Heating upon Glyoxalase Activity.

Determination of the effect of heat on glyoxalase resolves itself into the determination of the activity of heated enzyme on methylglyoxal. Heating the enzyme at 60° for 5 minutes causes a considerable diminution of activity, becoming much more marked at 80° and 95° (Table VIII).

TABLE VIII.

Influence of Heating upon Glyoxalase Activity. Decrease of Methylglyoxal.

0.0144 gm. of methylglyoxal + heated liver extract (= 2 gm. of liver) of pH 7.0 at 37.5°.

Incubation with methylglyoxal.	Liver extract. Time of heating, 5 min.			
	Not heated (control).	Heated at 60°.	Heated at 80°.	Heated at 95°.
	Decrease of methylglyoxal.			
hrs.	*per cent*	*per cent*	*per cent*	*per cent*
1.5	41.1	28.8	5.2	2.9
3.0	74.7	51.5	7.0	4.3
5.5	87+	80.1	12.4	7.5

TABLE IX.

Formation of Lactic Acid from Methylglyoxal by Glyoxalase.

0.072 gm. of methylglyoxal + tissue extract (= 2 gm. of liver).

Time of incubation.	Decrease of methylglyoxal.	Increase of lactic acid.
hrs.	*per cent*	*per cent*
0.5	15.2	11.4
1.0	32.6	31.9
1.5	39.4	40.5
2.5	65.5	63.5
3.5	83.0	80.8

5. Formation of Lactic Acid from Methylglyoxal by Glyoxalase.

To 10 cc. of 0.1 M methylglyoxal were added 10 cc. of the liver extract (52 hours old). The mixture was diluted to 100 cc. with borate-boric acid mixture of pH 7.5 and incubated at 37.5°. 10 cc. of the fluid were taken out at intervals, added to 4 cc. of 10 per cent trichloroacetic acid and 6 cc. of water, shaken well, and filtered after 10 minutes. 10 cc. of the filtrate were used for the

lactic acid determination and 5 cc. for the methylglyoxal determination. The control experiments were made by diluting 10 cc. of the extract to 100 cc. with the buffer to see whether there might be observed any increase of lactic acid from the liver extract during the incubation.

As shown in Table IX the liver extract converted methylglyoxal quantitatively to lactic acid. The control experiments showed no perceptible formation of lactic acid from the liver extract alone.

6. Formation of Lactic Acid from Methylglyoxal by Muscle.

The experiments were carried out with hashed muscle under the same conditions as those with liver extract.

Several experiments showed always insufficient formation of lactic acid in comparison with the decrease of methylglyoxal. The difference was, however, smaller with the decrease of methylglyoxal or the increase of lactic acid; as given in Table X, after 30 minutes incubation the formation of lactic acid was 54 per cent of the decrease of methylglyoxal, 79 per cent after 90 minutes, and 93 per cent after 150 minutes.

Small amounts of lactic acid, produced from muscle in the control experiments, were subtracted from the figures obtained in the main experiment; the figures in the third column show the difference.

7. Relative Glyoxalase Content of Animal Tissues.

1 gm. of fresh tissues was ground thoroughly with 3 gm. of clean sand and added to 10 cc. of 0.1 M methylglyoxal solution (neutralized). The whole volume was made up to 100 cc. with pH 7.0 borate-boric acid buffer solution which had been warmed at 37.5°. The above operation was performed within 10 minutes after killing the animals. After 20 minutes incubation at 37.5° the decrease of methylglyoxal was determined and the glyoxalase activity of the tissue was calculated from the amount of methylglyoxal thus converted. For comparison 1 unit of activity was arbitrarily taken as that of 1 gm. of tissue which converts 20 mg. of methylglyoxal (of the 90 mg. added) in 20 minutes under the conditions stated. For example, if after this brief period of incubation analysis of the aliquot of the trichloroacetic acid filtrate gave a

total of 80 mg. of methylglyoxal in the 100 cc. of incubated mixture, 10 mg. had been converted, and the activity is recorded as 0.5. The results are subject to considerable accumulated error, but show roughly the relative glyoxalase activity.

TABLE X.

Formation of Lactic Acid from Methylglyoxal by Muscle.

0.072 gm. of methylglyoxal + 2 gm. of muscle hash.

Time of incubation.	Decrease of methylglyoxal.	Increase of lactic acid.	Control. Lactic acid in 2 gm. muscle.
min.	*per cent*	*per cent*	*mg.*
0			11.1
30	17.8	9.6	13.1
90	84.5	67.0	13.5
150	91.0	85.0	13.5

TABLE XI.

Relative Glyoxalase Content of Animal Tissues.

Animal.	Experiment No.	Relative glyoxalase activity.*						
		Liver.	Brain.	Muscle.	Blood.	Kidney.	Spleen.	Lung.
Rabbit.	I	0.8	0.5	0.4	0.3	0.7		0.4
	II	1.2	0.5	0.5	0.4	0.5		0.4
	III	0.9	0.5	0.5	0.2	0.8	0.5	0.6
White rat.	I	1.0	0.5	0.3	0.3	0.3		0.5
	II	0.7	0.4	0.4	0.4	0.4		0.5
	III	0.6	0.3	0.2	0.2	0.2	0.2	0.2
Dog.	I	0.7	0.4	0.6	0.2	0.7	0.3	0.5

* The relative glyoxalase activity is given here as units, determined according to the procedure described in the text.

Judging from the results shown in Table XI, the liver is most active in each animal, the other tissues containing from one-third to two-thirds as much glyoxalase activity as the liver.

The relative glyoxalase content of fasting animals was also examined. In one series of experiments the glyoxalase content of the liver and the muscle of rabbits which had been fasted for 7 and 10 days was measured. In another series the same experiment

was performed, rabbits, which had been injected with 0.25 gm. of phlorhizin suspended in 2.5 cc. of olive oil every 24 hours for 3 days, being used. The conditions of the experiment were just the same as the previous ones. The results given in Table XII indicate that the glyoxalase content of the muscle decreased distinctly, while that of the liver remained unchanged.

8. Influence of Toluene on Activity of Glyoxalase.

Dakin and Dudley (8) observed a marked inhibitory action of toluene on the activity of glyoxalase. They added 2 cc. of toluene to 50 cc. of dog liver extract. After 2½ or 3 hours incubation, 0.2 gm. of phenylglyoxal was added and the rotation of mandelic acid formed from phenylglyoxal was examined.

TABLE XII.

Relative Glyoxalase Activity of Tissues of Fasted and Phlorhizinized Rabbits.

	Experiment No.	Glyoxalase activity. *	
		Liver.	Muscle.
Fasted rabbit.	I (fasted 7 days).	0.7	0.14
	II (" 10 ").	0.8	0.14
Phlorhizinized rabbit.	I	1.0	0.11
	II	0.8	0.12

* The relative glyoxalase activity is given here as units, determined according to the procedure described in the text.

In the present experiments the mixture of 10 cc. of liver extract (72 hours old), varying amounts of toluene (1.0, 0.5, 0.1, and 0.05 cc.), and 50 cc. of citrate buffer (pH 6.8) was incubated for 3 hours and then 10 cc. of 0.02 M methylglyoxal solution were added. After 2 more hours incubation the remaining methylglyoxal was determined, the results being compared with the control experiment which was carried out under the same conditions but without toluene.

No inhibitory action of toluene was observed. Thus the amount of toluene which we use for the antiseptic purpose seems to have no influence on the activity of glyoxalase.

9. Influence of Sodium Fluoride on Action of Glyoxalase.

It is a well known fact that fluoride markedly inhibits yeast fermentation or tissue glycolysis. The question, at which stage of the sugar catabolism fluoride acts, has, however, not been solved. Davenport and Cotonio (14) observed that in the presence of NaF only a small part of glycogen added was converted to lactic acid by the muscle extract while glycogen and free phosphate disappeared rather rapidly. This observation may indicate that the inhibition takes place after the stage of hexose phosphate formation. To see whether fluoride may have an inhibitory action on the reaction methylglyoxal → lactic acid, 2.5 cc. of the liver extract and 2 cc. of 0.1 M methylglyoxal were diluted with and without NaF (final concentration = 0.01 N) to 50 cc. with borate buffer of pH 7.0 and incubated at 37.5°.

The occasional determination of methylglyoxal and lactic acid of these two fluids showed that fluoride had no effect on glyoxalase. Its inhibition thus would appear to be located between hexose phosphate and methylglyoxal.

Other experiments showed that the accelerating action of arsenate and arsenite on yeast fermentation has no parallel on glyoxalase action.

IV. "Antiglyoxalase."

In Dakin and Dudley's experiments (8) the pancreas of several animal species showed, in striking contrast to other tissues, an inhibitory action on glyoxalase. This inhibitory substance, named by them "antiglyoxalase" was present not only in pancreas extract but also in pancreatic juice and pancreatin preparations. The amount of the enzyme, present in 0.025 gm. of pancreatin or 2 cc. of pancreatic juice was stated to inhibit completely the action of glyoxalase which otherwise would be capable of forming several gm. of lactic acid. Antiglyoxalase was thermolabile, being completely destroyed by exposure to a temperature of 85°, for 10 minutes. The inhibitory action was not due to trypsin, lipase, or diastase.

Foster and Woodrow (15) and Foster (10) found a substance in pancreas which inhibited the formation of lactic acid in chopped muscle. This pancreatic factor differed from antiglyoxalase in

its solubility in 70 per cent alcohol, stronger thermostability, and weaker resistance towards alkali. They proved that insulin and trypsin were not responsible for the inhibitory action. Foster suggested that the antiglyoxalase action might be due to the action of some factor in pancreas on the substrate (phenylglyoxal) rather than on glyoxalase itself.

1. Inhibitory Action of Pancreas Extract on Glyoxalase.

20 cc. of rabbit liver extract (some days old) were added to 16 cc. of 20 per cent beef pancreas extract (24 hours old), diluted to 100 cc. with citrate buffer of pH 6.8, and incubated at 37.5°. Occasionally 20 cc. were pipetted out of the fluid, added to 2 cc. of 0.1 M methylglyoxal, diluted to 50 cc. with the buffer, incubated

TABLE XIII.

Action of Pancreas Extract on Glyoxalase.

Time of contact of pancreas extract with glyoxalase.	Decrease of methylglyoxal after 2 hrs. incubation with glyoxalase + pancreas extract.	
		Control (without pancreas extract).
hrs.	*per cent*	*per cent*
0	17.2	11.9
1	14.8	12.0
3	6.1	11.0
5	3.0	10.0

for 2 hours, and examined for the remaining methylglyoxal. As a control experiment the liver extract was treated under the same conditions but without the pancreas extract.

The figures in Table XIII indicate that the pancreas extract must be kept in contact with glyoxalase for 2 or 3 hours before the addition of methylglyoxal to develop its inhibitory action. In the early stage of the incubation the pancreas extract had even an accelerating action on glyoxalase. This may be due to the presence of glyoxalase in the pancreas itself. The complete inhibition as described by Dakin and Dudley could not be observed even after 18 hours digestion of 7 gm. of beef pancreas and 2 gm. of rabbit muscle.

2. Inhibitory Action of Pancreatin Preparation.

The commercial pancreatin preparation (Park Davis and Company) had, contrary to the pancreas, some inhibitory action without previous incubation with the ferment (Table XIV).

Practically complete inhibition was observed when 25 cc. of liver extract were digested with 1 gm. of pancreatin at 33° for 18 hours at pH 7. It may be noted that pancreatin alone destroys methylglyoxal to a certain extent.

TABLE XIV.

Inhibitory Action of Pancreatin on Glyoxalase.

1. 2 cc. liver extract + 2 cc. 0.1 M methylglyoxal (no pancreatin).
2. 2 cc. liver extract + 2 cc. 0.1 M methylglyoxal + 0.01 gm. pancreatin.
3. 2 " " " + 2 " 0.1 " " + 0.025 " "
4. 2 " " " + 2 " 0.1 " " + 0.11 " "

Each diluted to 50 cc. with borate buffer solution, pH 7.

Time (+ methylglyoxal).	Decrease of methylglyoxal.			
	Sample 1.	Sample 2.	Sample 3.	Sample 4.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*
0.5	24.5	22.2	17.5	8.5
1.5	76.6	69.0	61.0	30.1

TABLE XV.

Decrease of Methylglyoxal.

Pancreas extract heated at 85°.	Pancreas extract heated at 100°.	Control.	
		With untreated pancreas extract.	Without extract.
per cent	*per cent*	*per cent*	*per cent*
8.2	8.2	6.1	13.6

3. Thermostability of Antiglyoxalase.

A. Pancreas.—The pancreas extract was heated for 10 minutes at 85° and for 15 minutes at 100° and treated similarly as in the foregoing experiment. The control experiments were made in the following way: The liver extract was treated with and without unheated pancreas extract under the same conditions as in the main experiment. Though the results are not very impressive, the pancreas loses its activity by heating (Table XV).

B. Pancreatin.—As shown in Table XVI heating did not destroy the inhibitory action completely and even after exposure to a temperature of 120° for 30 minutes the pancreatin still kept its inhibitory function. Judging from the result mentioned above the author has some doubt about the enzymic nature of the pancreatic factor.

4. Nature of Action of Pancreatic Factor.

Foster (10) is inclined to attribute the antiglyoxalase action to the action of some factor in the pancreas on the substrate rather than on the glyoxalase. To examine her suggestion the following experiments were performed, methylglyoxal being used instead of phenylglyoxal.

TABLE XVI.

1. 10 cc. liver extract + 2 cc. 0.1 M methylglyoxal.
2. 10 " " " + 15 " 2 per cent pancreatin (untreated).
3. 10 " " " + 15 " pancreatin (120°, 30 min.).

The other conditions are the same as in the experiment in Table XIV.

Time.	Decrease of methylglyoxal.		
	Sample 1.	Sample 2.	Sample 3.
hrs.	*per cent*	*per cent*	*per cent*
0.5	28.2	10.3	21.5
2.0	91+	25.5	62.5

Experiment A.—A mixture of 5 cc. of 0.02 M methylglyoxal and 5 cc. of pancreas extract was incubated for 3 hours, added to 5 cc. of liver extract (96 hours old), which had been warmed for 3 hours, and diluted to 50 cc. with citrate buffer solution of pH 6.8.

Experiment B.—The mixture of 5 cc. of the pancreas extract and 5 cc. of the liver extract was incubated for 3 hours, added to 5 cc. of methylglyoxal, and diluted to 50 cc. with the buffer solution.

Experiment C.—The mixture of 5 cc. of the liver extract and 5 cc. of water was incubated for 3 hours, added to 5 cc. of methylglyoxal and 5 cc. of the pancreatin solution, which had been warmed for 3 hours, and diluted to 50 cc. with the buffer solution.

Experiment D.—The mixture of 5 cc. of the liver extract and 5 cc. of water was incubated for 3 hours, added to 5 cc. of methylglyoxal, and diluted to 50 cc. with the buffer solution.

XUM

All fluids were placed in the thermostat at 37.5° for 2½ hours and then examined for their methylglyoxal content. The results are given in Table XVII.

If Foster's suggestion were right, we should have observed the inhibitory action of the pancreatic factor in Experiment A, which shows no special relation between methylglyoxal and pancreatic factor.

The absence of significant difference between the results of Experiments B and C indicates that the pancreatic factor inhibited the action of glyoxalase only when it had been kept in contact with glyoxalase for a time before the addition of methylglyoxal. The inference seems to be justified that the inhibitory action is directly upon the glyoxalase. That pancreatin inhibits the power of glyoxalase to convert methylglyoxal to lactic acid is shown in the following experiments.

TABLE XVII.

Nature of Action of Pancreatic Factor.

Decrease of methylglyoxal.			
Experiment A.	Experiment B.	Experiment C.	Experiment D.
per cent	*per cent*	*per cent*	*per cent*
17.1	11.4	19.1	18.5

Experiment 1.—10 cc. of 20 per cent liver extract + 5 cc. of 3 per cent pancreatin suspension + 15 cc. of borate-boric acid buffer of pH 7.4.

Experiment 2.—10 cc. of liver extract + 5 cc. of water + 15 cc. of buffer.

Experiment 3.—5 cc. of pancreatin +10 cc. of water + 15 cc. of buffer.

The mixtures were incubated at 37.5° for 3 hours, then added to 2 cc. of 0.2 M methylglyoxal, and diluted to 50 cc. with the same buffer. These mixtures were incubated further and the decrease of methylglyoxal and the increase of lactic acid were examined at suitable intervals (Table XVIII).

When the glyoxalase action of pancreatin on methyglyoxal (Experiment 3) is subtracted from the results in Experiment 1, the decrease of methylglyoxal and the increase of lactic acid

coincide. This suggests a direct inhibition of pancreatic factor on glyoxalase.

5. Action of Insulin on Glyoxalase.

Foster and Woodrow (15) found that the presence of insulin had no influence on the formation of lactic acid from chopped muscle. In the present experiment it was found that insulin (iletin U-80 and a sample prepared by Somogyi, Doisy, and Shaffer's method (16)) was entirely without effect on glyoxalase activity of liver extracts.

V. Action of Potassium Cyanide on Methylglyoxal and Glyoxal.

Meyerhof (13) has described the interesting observation that hydrogen cyanide, even in a very low concentration, catalyzes

TABLE XVIII.

Nature of Action of Pancreatic Factor.

Time.	Experiment 1.		Experiment 2.		Experiment 3.	
	Decrease of methylglyoxal.	Increase of lactic acid.	Decrease of methylglyoxal.	Increase of lactic acid.	Decrease of methylglyoxal.	Increase of lactic acid.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	29.0	24.0	40.0	38.0	5.0	0.5
3.4	44.6	36.6	92.9	83.4	12.0	1.2

the conversion of methylglyoxal to lactic acid in neutral reaction. A concentration of 5×10^{-5} M KCN converted 100 times that amount of methylglyoxal to lactic acid within 1 hour at 38°. He determined lactic acid *indirectly* from the quantity of carbon dioxide formed in Warburg's apparatus for glycolysis. At the same time the decrease of methylglyoxal was tested qualitatively by its reaction with piperidine and sodium nitroprusside.

Independently Dr. Shaffer had observed the striking effect of cyanide in activating the reducing intensity of glyoxals, which serves as the basis for our colorimetric method earlier described.

In view of these observations it was of interest to study the effect of cyanide on glyoxals.

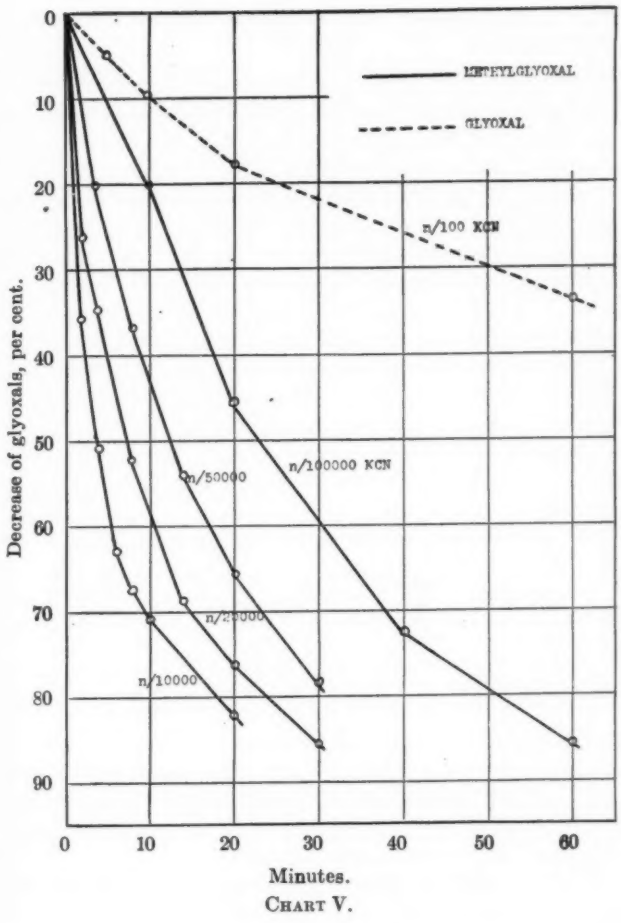

CHART V.

1. Action of KCN on Methylglyoxal and Glyoxal.

As illustrated in Chart V KCN had such a splendid action on methylglyoxal that even in 0.00001 N concentration it decomposed 86 per cent of 0.001 M methylglyoxal in an hour's time at 37.5° and at pH 6.8 (citrate), at which pH methylglyoxal without

cyanide is quite stable. The result shows that the action of KCN is catalytic.

Glyoxal is much more resistant to KCN than methylglyoxal.

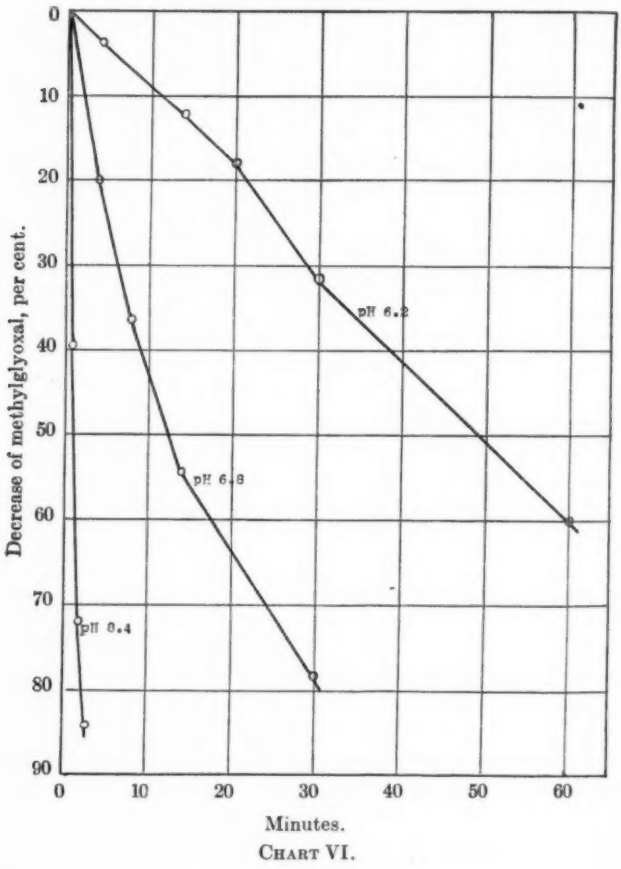

CHART VI.

The glyoxals disappeared with great velocity up to about 90 per cent and then the change became extremely slow. Meyerhof suggested that this phenomenon might be due to the formation of some unknown substance or substances.

2. Influence of Hydrogen Ion Concentration on Catalytic Action of KCN.

Chart VI shows the great increase of KCN action with the rise of hydroxyl ion concentration. In 0.00002 N KCN at 37.5° 3.8 per cent of 0.001 M methylglyoxal disappeared in 4 minutes at pH 6.2, 20.0 per cent at pH 6.8, and 100 per cent at 8.4. The results of the calculation of velocity constants of the reaction with 0.00002 N KCN at pH 6.8 conform fairly to a monomolecular reaction but those of the reactions with other amounts of KCN and at other pH values were not satisfactory enough for the application of monomolecular equation to them. Nevertheless, the average values of velocity constants may be used for approximate comparison of velocities of the decomposition of methylglyoxal by various agents (alkali, glyoxalase, or KCN).

Concentration of KCN.	pH	$K \times 10^2$ (log e)
M		
0.0001	6.8	16.1
0.00005	6.8	9.2
0.00002	6.8	5.5
0.00001	6.8	3.2
0.00002	6.2	1.2
0.00002	8.4	57.5

3. Lactic Acid Formation from Methylglyoxal in Presence of KCN.

Having confirmed the catalytic action of KCN upon glyoxals, we determined the amount of lactic acid formed from methylglyoxal by cyanide decomposition. At pH lower than 12 little or no lactic acid was found to be produced by this reaction. The attempt to prepare zinc lactate failed also. An inconsistent increase of the acidity of about 30 per cent was, however, observed in every case. Two typical results out of more than ten experiments with varying amounts of KCN may be cited (Table XIX).

The mixture of 20 cc. of 0.2 M methylglyoxal and 1 cc. of 0.01 N (final concentration = 0.0001 N) or 2 cc. of 0.1 N KCN (final concentration = 0.002 N) was diluted to 100 cc. with 0.1 M $NaHCO_3$ (pH about 8.4) at 22°. Methylglyoxal, lactic acid, and acidity were examined at intervals. A control experiment was

made in the same way but without KCN. For the determination of the acidity 10 cc. of the fluid were poured into 15 cc. of 0.1 N HCl, aerated rapidly with CO_2-free air for 2 to 3 minutes, and titrated with 0.1 N NaOH.

The failure to find lactic acid was proved not to be due to its further decomposition by KCN action.

To exclude the possibility of the oxidation of lactic acid (or a precursor) by oxygen of the air the mixture of methylglyoxal and KCN was made in a hydrogen atmosphere. No difference was

TABLE XIX.

Conversion of Methylglyoxal in Presence of KCN.

Time.	Decrease of methylglyoxal.		Increase of acidity.*		Increase of lactic acid.	
		Control without KCN.		Control without KCN.		Control without KCN.
	KCN = 0.0001 N.					
min.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
5	29.5	0				
10	58.9		22.5			
20	85.1		35.0			
30	88.4	3.6	35.0	0	0.6	0.4
	KCN = 0.002 N.					
5	41.3	0	27.0	0	0	0
10	66.4		28.0			
20	86.3		31.5			
30		2.0	31.5	0	0	0

* The calculation was made under the assumption that 1 molecule of a monobasic acid was formed from 1 molecule of methylglyoxal.

recognized in the results as against that obtained in the ordinary atmosphere. The experiments given in Table XX show that KCN inhibits even the action of high alkalinity in converting methylglyoxal to lactic acid and turns the reaction into another unknown direction. At the lowest concentration (0.00002 N) the KCN action was so weak that it scarcely checked the predominant action of alkali. 0.0001 N concentration was, however, strong enough to restrain lactic acid formation to a remarkable degree. With a sufficient amount of KCN (0.001 N) the increase

of the acidity was just 50 per cent of the decrease of methylglyoxal and no trace of lactic acid was observed.

4. Nature of Substance or Substances Produced from Methylglyoxal by KCN.

Since the formation of pyruvic acid and acetol from methylglyoxal, by a Cannizzaro reaction, seemed to be possible, the isolation of *p*-nitrophenylhydrazone of pyruvic acid from the solu-

TABLE XX.

Methylglyoxal Conversion by KCN.

10 cc. of 0.1 M methylglyoxal + 40 cc. of 0.1 N NaOH + varying amounts of KCN, diluted to 100 cc. with water. Temperature about 25°.

KCN concentration	0.00002 N			0.0001 N			0.0002 N			0.001 N		
Time.	Decrease of methylglyoxal.	Increase of acidity.	Increase of lactic acid.	Decrease of methylglyoxal.	Increase of acidity.	Increase of lactic acid.	Decrease of methylglyoxal.	Increase of acidity.	Increase of lactic acid.	Decrease of methylglyoxal.	Increase of acidity.	Increase of lactic acid.
min.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
0.5	20.8		17.6									
1.0				57.2			54.1					
1.5										100	50	0
2.0	54.8	49.2	36.0	69.4	62.5	21.9	79.2					
3				80.1			88.6	61.5	15.3			
4	62.4	57.5	45.5	86.1	71.0	24.7		63.5	16.4			
5								68.1	17.0			
6	73.1	65.8	57.7									
7					72.4	26.7			17.9			
10	87.6	81.4	64.8		77.5				19.5		50	0

tion was attempted, but failed. The absence of acetaldehyde was proved in the lactic acid determination method in the previous experiments. Ethyl alcohol was not detected. For the examination of the volatile acid the following experiment was performed. The mixture of 40 cc. of 0.47 M methylglyoxal and 25 cc. of 0.1 N KCN was diluted to 200 cc. with 0.2 M $NaHCO_3$ (pH about 8.4) and let stand overnight at about 23°. (Though hydrogen ion concentration of pH about 7 was desirable in the experiment,

bicarbonate was the only available buffer; citrate buffer, being oxidized by $KMnO_4$, interferes in the lactic acid determination and borate-boric acid buffer checks the action of KCN.) The amount of methylglyoxal that disappeared was 96.8 per cent and the increased acidity corresponded to 71.2 cc. of 0.1 N NaOH. The fluid was acidified with phosphoric acid and then distilled with steam. The acidity in the distillate (4 liters) was equivalent to 70.8 cc. of 0.1 N NaOH. The distillate was made slightly alkaline and evaporated to 100 cc. To remove formic acid, if present, the concentrated fluid was boiled with $K_2Cr_2O_7$-H_2SO_4 for 10 minutes according to Macnair's description (17) and again distilled with steam. The acidity of this distillate corresponded to 68.23 cc. of 0.1 N NaOH. Thus the presence of formic acid was excluded by no significant difference in the acidity before and after the oxidation with bichromate. The existence of acetic acid was expected but it was not proved positively. The distillate reduced Fehling's solution and ammoniacal silver nitrate (perhaps due to methylglyoxal remaining in a small quantity). The presence of formaldehyde and acetone was not excluded with certainty.

We have been unable so far to identify the product of the transformation of methylglyoxal under the catalytic influence of cyanide. The product is not lactic acid. One of the products, a volatile acid, has so far not been identified.

VI. Reducing Action of Methylglyoxal and Glyoxal when Acted upon by Glyoxalase, Alkali, and Potassium Cyanide.

The development of high reduction intensity during the action of glyoxalase or alkali on methylglyoxal would perhaps not be expected since the reaction, involving the formation of lactic acid from methylglyoxal, is not simple oxidation but oxidation-reduction. The circumstances may, however, be quite different in the case of KCN. The following experiment (Table XXI) was made to observe whether there would develop high reduction intensity when glyoxal and methylglyoxal are decomposed by KCN.

1. Methylglyoxal.

10 cc. of 0.1 M methylglyoxal solution were added with 3 cc. of 0.1 per cent methylene blue solution and 10 and 5 cc. of 0.001 N

KCN solution, so as to get the final concentration of 0.0001 N and 0.00005 N in 100 cc. The mixture was immediately diluted to 100 cc. with citrate buffer solution of pH 6.8. A small part of the fluid was transferred to the colorimeter tube, covered with mineral oil to prevent the reoxidation of the reduced methylene blue, and read at intervals against the standard which was made by diluting 3 cc. of methylene blue to 100 cc. with the buffer

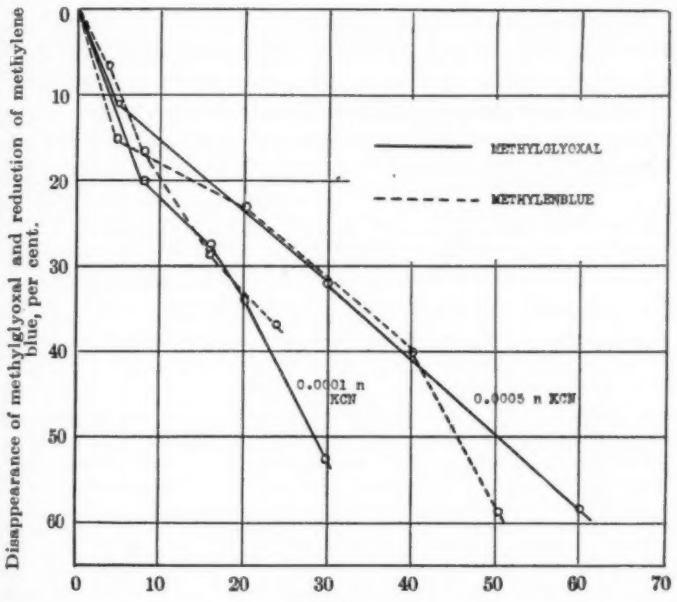

CHART VII.

solution. The temperature was about 25° throughout the experiment. The same experiment but without methylene blue was made for the determination of the rate of the decrease of methylglyoxal by the action of KCN. Control experiments were conducted by adding 3 cc. of methylene blue to 10 or 5 cc. of KCN solution and to 10 cc. of methylglyoxal for the examination of reducing power of KCN or methylglyoxal. Chart VII and Table

XXI show the development of high reduction intensity during the change of methylglyoxal by the action of KCN. The control experiments indicated no reduction of methylene blue by KCN or methylglyoxal alone.

2. Glyoxal.

The experimental conditions are similar to those of the previous experiment, the KCN concentration being 0.001 N.

High reducing action was thus observed with both glyoxal and methylglyoxal. In the similar experiments, using glyoxalase or

TABLE XXI.

Rate of Methylene Blue Reduction by Glyoxal + KCN.

Final concentrations: 0.01 M methylglyoxal + 0.001 N KCN + 0.003 per cent methylene blue; pH 6.8; temperature about 25°.

Time.	Decrease of methylglyoxal at pH 6.8 in 0.001 N KCN.	Reduction of methylene blue.		
		Methylglyoxal + methylene blue + KCN.	Control.	
			Methylglyoxal + methylene blue.	KCN + methylene blue.
min.	*per cent*	*per cent*	*per cent*	*per cent*
5	5.0	23.3	1.0	0
10	9.5	30.0	2.5	0
20	17.7	48.1	4.5	0
30		67.1		0
35			9.5	0
50	34.4	77.6		0
60			11.0	0
90	44.5	80.3		0

alkali instead of KCN, we could not notice even a slight degree of reducing action on methylene blue. This contrast suggests a different mode of the action of KCN and glyoxalase or alkali on glyoxals.

It is the author's pleasure to express sincere gratitude to Professor P. A. Shaffer for his valuable suggestions and criticism throughout the experiments.

SUMMARY.

1. A new colorimetric method for the determination of glyoxals is described.

2. The influence of hydrogen ion concentration on glyoxals is studied; the glyoxals are quite stable at neutral reaction but with the increasing pH they disappear more rapidly at rates of monomolecular reactions. The disappearance of glyoxals, however, at a pH lower than pH 12 does not necessarily mean the formation of corresponding hydroxy acids. The conversion of methylglyoxal to lactic acid is quantitative only in the excess of alkali.

3. The action of tissue extracts (glyoxalase) on glyoxals was studied. The optimum hydrogen ion concentration for the activity of glyoxalase lies in the neighborhood of pH 7. The enzyme converts methylglyoxal quantitatively to lactic acid. The glyoxalase content of liver is higher than in other animal tissues.

4. The inhibitory action of pancreas extracts upon glyoxalase has been further investigated. Insulin does not exhibit any inhibitory action.

5. Alkali cyanide has strong catalytic action on the decomposition of glyoxals. Lactic acid is not produced from methylglyoxal in the presence of KCN.

6. High reduction intensity develops when glyoxals are destroyed by KCN but not when decomposed by glyoxalase or alkali.

Addendum.—While the present papers were under preparation for publication, Kuhn and Heckscher (18) reported a work on methylglyoxal. They studied the behavior of methylglyoxal towards the OH ion concentration and glyoxalase, using the iodometric method for methylglyoxal determination. Their results conform in principle to ours.

BIBLIOGRAPHY.

1. Benedict, S. R., *J. Biol. Chem.*, 1922, li, 189.
2. Walker, E., *Biochem. J.*, 1925, xix, 1082.
3. de Forcrand, *Bull. Soc. chim. de Paris*, 1884, xli, 240; cited by Houben-Weyl, Methoden der organische Chemie, Leipsic, 1922, ii, 6.
4. Fischer, H. O. L., and Taube, C., *Ber. chem. Ges.*, 1924, lvii, 1502.
5. Friedemann, T. E., *J. Biol. Chem.*, 1927, lxxiii, 331.
6. Shaffer, P. A., and Friedemann, T. E., *J. Biol. Chem.*, 1924, lxi, 585.
7. Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, lxxiii, 335.
8. Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xiv, 155, 423.
9. Neuberg, C., *Biochem. Z.*, 1913, xlix, 502; 1913, li, 484.
10. Foster, D. L., *Biochem. J.*, 1925, xix, 757.

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11. Dudley, H. W., *Biochem. J.*, 1926, xx, 314.
12. Morris, J. L., and Macleod, A. G., *J. Biol. Chem.*, 1922, l, 55.
13. Meyerhof, O., *Biochem. Z.*, 1925, clix, 432.
14. Davenport, H. A., and Cotonio, M., *J. Biol. Chem.*, 1927, lxxiii, 463.
15. Foster, D. L., and Woodrow, C. E., *Biochem. J.*, 1924, xviii, 562.
16. Somogyi, M., Doisy, E. A., and Shaffer, P. A., *J. Biol. Chem.*, 1924, lx, 31.
17. Macnair, D. S., *Z. anal. Chem.*, 1888, xxvii, 398.
18. Kuhn, R., and Heckscher, R., *Z. physiol. Chem.*, 1926, clx, 116.

THE FORMATION OF METHYLGLYOXAL FROM HEXOSE PHOSPHATE IN THE PRESENCE OF TISSUES.

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In a paper by Toenniessen and Fischer (1) the important observation is reported that methylglyoxal is formed from hexose phosphate when the latter is digested with muscle in the presence of pancreas. They identified the substance by determining the melting point of its *p*-nitrophenylosazone. The significant point in their work seems to be their use of pancreas which by its antiglyoxalase might retard the further destruction of methylglyoxal, if this substance were produced from hexose phosphate by the tissue, thus allowing the methylglyoxal to accumulate in the tissues or solution. Since this work seemed to be of great significance in confirmation of the notion that methylglyoxal may be an intermediate between hexose phosphate and lactic acid, an attempt was made to repeat the work. The hexose phosphate used was sodium hexose diphosphate formed from calcium salt (candiolin)[1] by treatment with sodium oxalate.

Experiment I.

Mixture 1.—2 gm. of fresh rabbit muscle + 0.5 gm. of pancreatin + 70 cc. of 0.65 per cent sodium hexose phosphate.

Mixture 2.—2 gm. of muscle + 70 cc. of hexose phosphate.

Mixture 3.—70 cc. of hexose phosphate.

Mixture 4.—2 gm. of muscle.

The mixtures were diluted to 100 cc. with borate-boric acid buffer of pH 7.0, incubated at 38°, and examined for their methylglyoxal and free phosphate content (see Table I).

[1] For a supply of candiolin (calcium hexose diphosphate) we are indebted to the Winthrop Chemical Company.

XUM

Methylglyoxal was determined by the colorimetric method (2), it being assumed that the blue color is due to the presence of methylglyoxal, and free phosphate by Briggs' method (3), in both cases after the removal of protein with trichloroacetic acid.

In a second experiment in which rabbit liver was used instead of muscle, the increase of methylglyoxal was 3.23 mg. in Mixture 1 and 4.47 in Mixture 2 after 24 hours incubation. These results show that the glyoxal-like substance increases somewhat after a few hours incubation with hexose phosphate. The presence of the pancreatin had, contrary to expectation, even a slight interfering influence on the color increase; the pancreatin apparently inhibits or destroys not only the glyoxalase but also the ferment which converts hexose phosphate to methylglyoxal. If we assume that the increase of the color is due to methylglyoxal formed

TABLE I.

Increase of Methylglyoxal and Free Phosphate from Hexose Phosphate by Muscle.

Time of incubation.	Mixture 1.		Mixture 2.		Mixture 3.		Mixture 4.	
	Methylglyoxal.	Phosphate.	Methylglyoxal.	Phosphate.	Methylglyoxal.	Phosphate.	Methylglyoxal.	Phosphate.
hrs.	*mg.*	*mg. P*	*mg.*	*mg. P*	*mg.*	*mg. P*	*mg.*	*mg. P*
1	0.02	0.03	0.27	0.34	0	0	0	0
2	0.70		1.27	1.36	0	0	0	0
4.5	3.17	3.55	4.38	4.12	0	0	0	0

(though this is not the only substance responsible for the color development—some other intermediary substances may possibly give the color), the formation of methylglyoxal can hardly be understood, for the glyoxalase of muscle or liver is so active that methylglyoxal formed from hexose phosphate must be rapidly converted to lactic acid and the accumulation of methylglyoxal would not be expected. A possible explanation of the observed facts might be the assumption that glyoxalase activity is destroyed rather rapidly during the incubation, while the enzyme which converts hexose phosphate to methylglyoxal survives for a longer time. To test this hypothesis the following experiments were performed. It was found that by the incubation of the liver with 5 parts of buffer solution of pH 7, or water, the glyoxalase

was destroyed rather rapidly. When such old extracts were used, in which glyoxalase activity had largely disappeared, there was observed a much greater increase in the glyoxal-like substance on incubation with hexose phosphate.

Experiment II.

50 cc. of 20 per cent liver extract were diluted to 200 cc. with borate-boric acid buffer (pH 7); 3 cc. of toluene were added and incubated at 37°. From time to time 25 cc. were pipetted out, added to 5 cc. of 0.04 M methylglyoxal, diluted to 50 cc. with the buffer, and incubated at 37°. The rate of disappearance of glyoxal represents the glyoxalase activity.

The influence of the period of autolysis of the liver on the yield of methylglyoxal is shown in Table II.

TABLE II.

Influence of Incubation of Liver Extracts on Their Glyoxalase Activity.

Time of incubation with methylglyoxal.	Per cent of methylglyoxal destroyed.					
	Period of autolysis of liver.					
	At start.	2 hrs.	6 hrs.	24 hrs.	48 hrs.	72 hrs.
15 min.	69.0	45.0				
30 "			58.0	4.6		
6 hrs.					1.7	0

Experiment III.

Fresh rabbit liver was incubated with 5 parts of borate-boric acid buffer (pH 7) and some toluene at about 37°. At the start and again after 24 hours incubation 10 cc. of the extract were pipetted out, added with 50 cc. of 1.2 per cent sodium hexose diphosphate solution, diluted to 100 cc., and incubated for 20 hours with 1 cc. of toluene. The liver extract, incubated for 24 hours before being added to hexose diphosphate, yielded 16.5 mg. of methylglyoxal, while 3.0 mg. were found in the case of the fresh liver extract.

This experiment, which has been repeated several times with similar results, seems to show that liver extracts, after incubation for a period with toluene, during which the glyoxalase activity is

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largely destroyed, still possess the ability to form from hexose diphosphate a substance which reacts with the arsenophosphotungstic acid reagent like a glyoxal, and is presumably methylglyoxal.

It must be stated that a larger amount of the tissues does not always yield a larger amount of methylglyoxal from the same quantity of hexose diphosphate. This may be due to the action of residual glyoxalase changing methylglyoxal to lactic acid.

To learn whether the substance which gave the blue color with the complex tungstic acid reagent was actually methylglyoxal, the attempt was made to prepare its *p*-nitrophenyldihydrazone and to determine its melting point.

Experiment IV.

To a neutralized trichloroacetic acid filtrate from a mixture of liver extract and hexose phosphate, incubated under conditions favorable for methylglyoxal formation (which solution contained according to colorimetric determination about 16.5 mg. of methylglyoxal (Experiment III), there was added double the calculated amount of *p*-nitrophenylhydrazine in acetic acid solution. After a few seconds at room temperature there occurred a bulky, red precipitate, which was filtered after several hours and washed thoroughly with water. Then the precipitate was boiled with *p*-nitrophenylhydrazine-acetic acid solution to convert the monohydrazone, if any, to dihydrazone. An alcoholic solution of the dihydrazone gave blue or violet color on the addition of alkali, while the monohydrazone gives a red or brown color. This test was described by Dakin and Dudley (4) as a sharp reaction for the detection of dihydrazone. The dihydrazone was filtered, washed with dilute acetic acid and water, and dissolved in the smallest possible amount of pyridine. Water was added to the red pyridine solution till a slight precipitation occurred. Then the solution was heated to dissolve the precipitate and allowed to cool slowly. Gradually the red hydrazone crystallized in long and thin needles. The crystals were filtered by suction, washed with water, and dried. The osazone of pure methylglyoxal was prepared in the same way. A part of the purified osazone of the two sources was recrystallized also from butyl alcohol. The osazones of methylglyoxal and the substance under examina-

tion melted side by side at 282–283° (uncorrected), becoming brown and softening at 276–277°. A mixture of both osazones melted also at 282–283°. Upon being heated rapidly they melted at about 300°. In the literature we find conflicting reports on the melting point of methylglyoxal *p*-nitrophenylosazone; 280° by Toenniessen and Fischer (1), and 302–304° by Dakin and Dudley (4). The discrepancy of the reports may be ascribed to the rate at which the osazone was heated. The fact that the osazone of the substance being examined has the same properties and the same melting point as those of pure methylglyoxal indicates that the substance formed from hexose phosphate by liver extract is really methylglyoxal. A small amount of a brown precipitate came down after a long period of time from hexose phosphate solution (without tissue) when treated with *p*-nitrophenylhydrazine. The brown precipitation occurred in a small quantity also in the solution from liver extract (without hexose phosphate). Both precipitates showed no definite crystal form under the microscope and did not come out from pyridine solution on the addition of water. The substance formed from hexose phosphate by liver extract was destroyed by glyoxalase and by alkali in the same way as methylglyoxal.

Since at least two enzymes seem to participate in the formation of lactic acid from hexose phosphate, the one converting hexose phosphate to methylglyoxal, the other, glyoxalase, changing methylglyoxal to lactic acid, an experiment was performed to learn whether heated tissue extract might still convert hexose phosphate to methylglyoxal.

Experiment V.

10 cc. of the liver extract which had been incubated at 37° for 72 hours, were heated in the boiling water bath for 15 minutes, added to 25 cc. of 1.15 per cent hexose phosphate solution, diluted to 50 cc. with borate-boric acid buffer of pH 7.0, and then incubated at about 36° for 24 hours.

As a control experiment the unheated liver extract was treated in the same way. The increase of methylglyoxal by the heated extract was 0.55 mg., while 2.86 mg. were obtained by unheated liver extract. The marked decrease of the tissue activity by

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heating indicates that the substance which converts hexose phosphate to methylglyoxal is of enzymic nature.

The following experiments indicate that longer incubation of the liver yielded more methylglyoxal but after 48 hours incubation the rate of the increase of methylglyoxal became rather slow. As to the duration of the digestion of hexose phosphate with the liver, methylglyoxal increased relatively rapidly till 48 hours and then slowly. Extracts of liver incubated with water yielded more methylglyoxal than extracts prepared by extraction and incubating with borate-boric acid buffer of pH 7.0 (Table III).

TABLE III.

Relation between Period of Incubation before and after Addition of Hexose Phosphate on Methylglyoxal Yield.

Time of digestion of hexose phosphate with liver.	Period of incubation before addition of hexose phosphate.							
	At start.		After 20 hrs.		After 44 hrs.		After 68 hrs.	
	Water.	Buffer.	Water.	Buffer.	Water.	Buffer.	Water.	Buffer.
	Increase of methylglyoxal, mg.							
hrs.								
4	0.08	0.07	0.66	0.31	1.11	0.97	0.65	0.96
8	1.16	0.76	2.65	1.10	3.22	3.29	3.45	3.58
24			5.51	2.40	10.51	5.68	11.95	8.29
48	5.67	2.87	8.17	5.96	16.36	6.48	12.73	
72	6.08	4.55						
96			9.25	6.74	17.60			

Experiment VI.

20 per cent extracts of fresh rabbit liver were made with (1) borate-boric acid buffer (pH 7) and (2) water, and incubated with some toluene at about 38°. At suitable intervals 10 cc. of each extract were taken out and added to 50 cc. of 1 per cent sodium hexose phosphate, diluted to 100 cc. with water, incubated with 1 cc. of toluene, and the increase of methylglyoxal in the solutions was examined by the colorimetric method.

No color increase was observed in hexose phosphate solution without liver. The difference in the methylglyoxal yield of these two kinds of liver extract (water and buffer) is, as shown in Experiment VIII, not due to the nature or hydrogen ion concentration of the extraction fluid but is merely due to hydrogen

ion concentration at which the extracts acted on hexose phosphate. In order that the methylglyoxal formed from hexose phosphate under the action of tissue extract may accumulate to an extent that its detection is possible, it appears to be necessary first to destroy the glyoxalase activity of the extract. This, as shown by the results of Experiment VII, is accomplished by incubation of the extract before it is treated with hexose phosphate. If the preliminary incubation is too much prolonged, however, the subsequent yield of methylglyoxal is small.

In an attempt to find the best conditions for the activity of the enzyme which is supposed to produce methylglyoxal from hexose phosphate, the influence of hydrogen ion concentration on methylglyoxal formation was studied.

TABLE IV.

Influence of pH of Extraction Fluid on Methylglyoxal Formation from Hexose Phosphate.

Time of incubation.	Increase of methylglyoxal, mg.			
	pH 5.0	pH 6.2	pH 7.0	pH 8.2
hrs.				
24	2.6	3.5	6.5	8.2
48	5.7	10.1	10.3	11.9
72	5.6	10.1	11.3	10.3

Experiment VII.

50 gm. of beef liver were incubated (32–35°) with 200 cc. of buffer solutions at pH 5.0 (citrate), 6.2 (citrate-NaOH), 7.0 (borate-boric acid), and 8.2 (borate-boric acid), and several cc. of toluene. After 24 hours 10 cc. of these extracts were added to 10 cc. of 3.8 per cent hexose phosphate, 80 cc. of pH 7.0 borate-boric acid buffer solution, and 1 cc. of toluene and incubated further. The increase of the blue color which develops upon adding the methylglyoxal reagents to the protein-free liquid is calculated in terms of methylglyoxal and shown in Table IV.

A control experiment showed that no trace of methylglyoxal was formed from hexose phosphate without the liver at any pH used in the experiment. It may be worth while to pay attention to the nature of the components of the buffer, since they them-

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heating indicates that the substance which converts hexose phosphate to methylglyoxal is of enzymic nature.

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	At start.		After 20 hrs.		After 44 hrs.		After 68 hrs.	
	Water.	Buffer.	Water.	Buffer.	Water.	Buffer.	Water.	Buffer.
	Increase of methylglyoxal, mg.							
hrs.								
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8	1.16	0.76	2.65	1.10	3.22	3.29	3.45	3.58
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48	5.67	2.87	8.17	5.96	16.36	6.48	12.73	
72	6.08	4.55						
96			9.25	6.74	17.60			

Experiment VI.

20 per cent extracts of fresh rabbit liver were made with (1) borate-boric acid buffer (pH 7) and (2) water, and incubated with some toluene at about 38°. At suitable intervals 10 cc. of each extract were taken out and added to 50 cc. of 1 per cent sodium hexose phosphate, diluted to 100 cc. with water, incubated with 1 cc. of toluene, and the increase of methylglyoxal in the solutions was examined by the colorimetric method.

No color increase was observed in hexose phosphate solution without liver. The difference in the methylglyoxal yield of these two kinds of liver extract (water and buffer) is, as shown in Experiment VIII, not due to the nature or hydrogen ion concentration of the extraction fluid but is merely due to hydrogen

ion concentration at which the extracts acted on hexose phosphate. In order that the methylglyoxal formed from hexose phosphate under the action of tissue extract may accumulate to an extent that its detection is possible, it appears to be necessary first to destroy the glyoxalase activity of the extract. This, as shown by the results of Experiment VII, is accomplished by incubation of the extract before it is treated with hexose phosphate. If the preliminary incubation is too much prolonged, however, the subsequent yield of methylglyoxal is small.

In an attempt to find the best conditions for the activity of the enzyme which is supposed to produce methylglyoxal from hexose phosphate, the influence of hydrogen ion concentration on methylglyoxal formation was studied.

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Experiment VII.

50 gm. of beef liver were incubated (32–35°) with 200 cc. of buffer solutions at pH 5.0 (citrate), 6.2 (citrate-NaOH), 7.0 (borate-boric acid), and 8.2 (borate-boric acid), and several cc. of toluene. After 24 hours 10 cc. of these extracts were added to 10 cc. of 3.8 per cent hexose phosphate, 80 cc. of pH 7.0 borate-boric acid buffer solution, and 1 cc. of toluene and incubated further. The increase of the blue color which develops upon adding the methylglyoxal reagents to the protein-free liquid is calculated in terms of methylglyoxal and shown in Table IV.

A control experiment showed that no trace of methylglyoxal was formed from hexose phosphate without the liver at any pH used in the experiment. It may be worth while to pay attention to the nature of the components of the buffer, since they them-

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selves may have some influence on the enzyme activity. A glance at Table IV indicates that the incubation of the liver with higher pH buffer solution produces a larger amount of methylglyoxal. But it must be considered that during the long incubation with relatively high pH buffer solution (over pH 8) a part of the methylglyoxal formed may be destroyed by alkalinity of the buffer. A pH of about 7 appears to be optimum during the incubation of the liver.

The next thing was to examine the optimum hydrogen ion concentration for the activity of the presumptive enzyme.

Experiment VIII.

To each portion of 10 cc. (from 2 gm. of liver) of 100 hours old 20 per cent watery extract of rabbit liver were added 10 cc. of 3.8 per

TABLE V.

Influence of pH on Methylglyoxal Formation.

Time of incubation of liver with hexose phosphate.	Increase of methylglyoxal, mg.			
	pH 5.0	pH 6.2	pH 7.0	pH 8.2
hrs.				
24	1.5	1.9	0.8	0.8
48	2.1	10.8	6.3	6.3
72	3.0	16.4	9.4	8.8

cent sodium hexose phosphate solution and 80 cc. of pH 5.0, 6.2, 7.0, and 8.2 buffer solutions respectively. They were placed with 1 cc. of toluene in the warm room (32–35°). From time to time small portions of the liquids were pipetted out and their methylglyoxal content examined.

Table V shows clearly that pH 6.2 buffer is the most favorable for the appearance of methylglyoxal.

As to the quantitative yield of methylglyoxal from hexose phosphate, it is difficult to draw very satisfactory conclusions. The complete destruction of glyoxalase by the incubation is apparently impossible without destroying also the enzyme which is presumed to change hexose phosphate to methylglyoxal. Under the best conditions the yield of methylglyoxal found was about 10 per cent of the calculated amount derivable from added hexose phosphate.

It has been observed that sodium fluoride inhibits markedly the action of the tissue to convert glycogen to lactic acid, leaving phosphate activity unattacked (5–8), and that it has no influence on glyoxalase (2). With the expectation that NaF might interfere in the reaction hexose phosphate → methylglyoxal, the following experiment was performed.

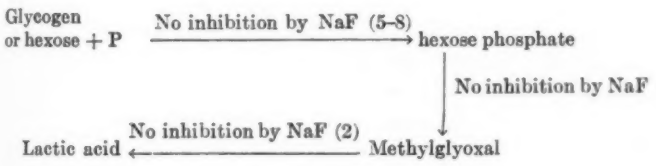
Experiment IX.

Mixture 1.—25 cc. of 1 per cent sodium hexose phosphate + 2 gm. of rabbit muscle hash (incubated for 30 hours).

Mixture 2.—25 cc. of hexose phosphate + 2 gm. of muscle hash + 1 cc. of 1 N NaF.

The solutions were diluted to 100 cc. and incubated with 1 cc. of toluene at about 38° for 21 hours. The increase of methylglyoxal was determined by the colorimetric method. The increase of methylglyoxal was 1.73 mg. without NaF and 2.01 mg. with NaF.

From the results it may be concluded that NaF was quite indifferent to the methylglyoxal formation from hexose phosphate. Such a conclusion however leaves the inhibitory action of NaF as a puzzle, because there appears to be no room for NaF action in the reactions glycogen → lactic acid:



SUMMARY.

1. When a mixture of hexose phosphate and muscle or liver extract, the tissue extract having been previously incubated with toluene for 24 hours, is incubated for another day, the solution contains a substance which reacts like methylglyoxal.

2. The identity of this substance with methylglyoxal appears to be established by the determination of the melting point of *p*-nitrophenyldihydrazone. The behavior of this substance to-

wards strong alkali and glyoxalase likewise confirms its identity with methylglyoxal.

3. Under the best conditions attained in the present work, the yield of methylglyoxal from hexose phosphate is about 10 per cent of the theoretical amount.

4. The explanation suggested is that the glyoxalase activity of the tissue having been destroyed by incubation, the methylglyoxal, formed from hexose phosphate by a somewhat more resistant enzyme, accumulates in the solution. The observed facts support the view that methylglyoxal may be an intermediate in lactic acid formation.

BIBLIOGRAPHY.

1. Toenniessen, E., and Fischer, W., *Z. physiol. Chem.*, 1926, clxi, 254.
2. Ariyama, N., *J. Biol. Chem*, 1928, lxxvii, 359.
3. Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.
4. Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv, 127.
5. Embden, G., and Lehnartz, E., *Z. physiol. Chem.*, 1924, cxxxiv, 273.
6. Embden, G., and Hayman, C., *Z. physiol. Chem.*, 1924, cxxxvii, 154.
7. Beattie, F., and Milroy, T. H., *J. Physiol.*, 1925, lx, 379; 1926, lxii, 174.
8. Davenport, H. A., and Cotonio, M., *J. Biol. Chem.*, 1927, lxxiii, 463.

THE COLORIMETRIC DETERMINATION OF LIPOID PHOSPHORUS IN BLOOD.

By ALVIN R. HARNES.

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(Received for publication, February 28, 1928.)

The colorimetric determination of phosphorus published by Briggs[1] lends itself to the determination of lipoid phosphorus without the oxidation of the organic substances present. In the following procedure the lipoid phosphorus is converted by hydrolysis with the H_2SO_4 present in Briggs' molybdate solution into the inorganic form of H_3PO_4. At the same time, the blue color of reduced molybdate is developed. The small amount of lipoid material remaining does not interfere with the reading of the color developed against the standard.

Procedure.—1 cc. of oxalated blood, serum, or plasma is spread on fat-free filter paper. The filter paper is cut in strips of $1\frac{1}{4} \times 7$ inches. The blood is dried in an electric oven at 50°. The strip containing the dried blood is then placed in a Folin sugar tube into which have been introduced about 4 cc. of chloroform. A test-tube, through which water is allowed to syphon, serves as a condenser. As many units as necessary may be connected (Fig. 1). The chloroform is then refluxed on the water bath at 75° for 3 hours. After the extraction is complete, the chloroform is transferred to a 50 cc. volumetric flask, diluted with 20 cc. of distilled water, and 3 cc. each of molybdate and hydroquinone solutions added.[2] The volumetric flask is placed in the water

[1] Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.

[2] *Molybdate Solution.*—5 per cent ammonium molybdate in 5 N H_2SO_4. Dissolve 25 gm. of ammonium molybdate in 300 cc. of H_2O, add 75 cc. of concentrated H_2SO_4, and dilute with 125 cc. of H_2O. *Hydroquinone Solution.*—Dissolve 5 gm. of hydroquinone and 25 gm. of $KHSO_3$ in 500 cc. of H_2O. *Standard Phosphorus Solution.*—Dissolve 219.3 mg. of KH_2PO_4 in 200 cc. of water and make up to 1 liter. 1 cc. of standard solution is equivalent to 0.05 mg. of phosphorus.

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FIG. 1.

TABLE I.

Results are expressed in mg. of phosphorus per 100 cc. of blood.

Blood sample No.	Evaporation of $CHCl_3$ and oxidation with H_2SO_4 and HNO_3.	Author's modification.
1	4.63	4.68
2	4.01	4.05
3	4.01	4.16
4	4.17	4.21
5	3.98	4.23
6	4.14	4.21
7	5.03	5.13
8	4.31	4.41
9	4.26	4.32
10	3.94	4.04

bath at 100° for 30 minutes, at which time the color is fully developed, and after cooling is read against the standard. This procedure eliminates the evaporation of the chloroform extract to dryness.

By making up the chloroform extract to 5 cc. and taking an aliquot part for the determination of phosphorus, cholesterol may be determined on the remaining portion.

Table I gives a comparison of results obtained from ten rabbit bloods.

The results thus far obtained indicate that when applied to the determination of lipoid phosphorus on small amounts of blood, the method described gives results that are as accurate as those obtained by other methods now in use. In addition, there is a great saving of time and material.

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COLORIMETRIC DETERMINATION OF IRON AND HEMOGLOBIN IN BLOOD. II.

By SAN YIN WONG.

(From the Department of Biochemistry, University of Hongkong, Hongkong, China.)

(Received for publication, March 12, 1928.)

In order to obviate the digestion for the total destruction of blood proteins required by my former method (1)—an inconvenience frequently met in the field and other places without a ready supply of gas—I have worked out another simple and rapid method which dispenses with heating entirely. In the latter method, the iron of the hemoglobin molecule is detached by the action of concentrated sulfuric acid as ordinarily done in the preparation of acid hematoporphyrin, the decomposition being facilitated with the aid of potassium persulfate. The interfering proteins are completely precipitated by tungstic acid and filtered off. Since the whole process involves no boiling, the concentration of acid in the unknown can be controlled absolutely and made exactly the same as in the standard for the production of color. The color developed is kept from fading away a much longer time than before by adding a quantity of potassium persulfate solution both to the unknown and to the standard.

The new method is as follows:

Reagents Required.

All the reagents except the standard must be iron-free as shown by blank tests.

1. *Concentrated Sulfuric Acid.*

2. *Sodium Tungstate.*—Dissolve an appropriate amount of good grade sodium tungstate to make a 10 per cent solution.

3. *Saturated Potassium Persulfate.*—Introduce into a small glass-stoppered bottle about 7 gm. of pure potassium persulfate and shake up with 100 cc. of distilled water. The undissolved

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portion settles on the bottom to make good any decomposition upon standing.

4. Potassium Sulfocyanate.—Prepare approximately a 3 N solution by dissolving 146 gm. of pure potassium sulfocyanate in distilled water to make 500 cc. Filter if necessary. Add 20 cc. of pure acetone to improve its keeping quality.

5. Standard Iron Solution.—Weigh out accurately 0.7 gm. of crystallized ferrous ammonium sulfate and dissolve in about 50 cc. of distilled water. Add to the solution 20 cc. of dilute (10 per cent) iron-free sulfuric acid, warm slightly, and then add 0.1 N (approximate) potassium permanganate solution to oxidize the ferrous salt completely. Dilute with distilled water to 1 liter exactly. Each cc. will contain 0.1 mg. of iron for use as a regular standard. To make weaker standards, dilute this standard solution accordingly.

Procedure.

Transfer accurately with an Ostwald pipette 0.5 cc. of blood into a 50 cc. volumetric flask and introduce 2 cc. of iron-free concentrated sulfuric acid. Whirl the flask to agitate the mixture for 1 or 2 minutes. Add 2 cc. of saturated potassium persulfate solution and shake. Dilute to about 25 cc. with distilled water and add 2 cc. of 10 per cent sodium tungstate solution. Mix. Cool to room temperature under the tap and then dilute to volume with distilled water. Stopper the flask and invert two or three times to effect thorough mixing. Filter through a dry filter paper into a clean, dry receiving vessel. Pipette exactly 20 cc. of the clear filtrate into a large test-tube graduated at 20 and 25 cc.

Measure into another similar test-tube exactly 1 cc. of the standard iron solution containing 0.1 mg. of Fe per cc. Add with a graduated 1 cc. pipette 0.8 cc. of iron-free concentrated sulfuric acid and dilute to the 20 cc. mark with distilled water. Cool to room temperature under the tap. Now add to both the unknown and standard 1 cc. of saturated potassium persulfate and 4 cc. of 3 N potassium sulfocyanate solution. Insert a clean rubber stopper, mix, and compare in a Duboscq colorimeter.

Calculation.—As the 20 cc. of filtrate taken represent 0.2 cc. of the original blood, and the quantity of standard solution used contains 0.1 mg. of Fe, if the reading is made with the standard set

at 20 mm., then 20 divided by the reading (R) of the unknown and multiplied by 50 will give the number of mg. of Fe in 100 cc. of the blood examined. To obtain the percentage of hemoglobin, divide this number by 3.35, since hemoglobin contains 0.0335 per cent of iron (2, 3).

$$\frac{20}{R} \times 50 = \text{mg. of Fe per 100 cc. of blood.}$$

$$\frac{20 \times 50}{R \times 3.35} = \text{percentage of hemoglobin in blood.}$$

TABLE I.

Comparative Determination of Iron in Blood by Wolter's Volumetric Method and by the New Colorimetric Method.

Source.	Volumetric method.	Colorimetric method.
	mg. per 100 cc.	*mg. per 100 cc.*
Pig 1	40.6	40.5
" 2	37.6	37.6
Ox 1	50.5	50.2
" 2	48.2	48.4
" 3	48.7	48.5
Chicken 1	35.4	35.4
" 2	37.6	37.6

TABLE II.

Determination of Iron in Solutions of Pure Methemoglobin of Dog.

Solution No.	Concentration.	Fe found.	Fe calculated.
	per cent	*mg. per 100 cc.*	*mg. per 100 cc.*
1	2.5	8.37	8.38
2	3.0	10.07	10.05
3	3.5	11.72	11.74
4	4.0	14.40	14.40

Accuracy.—Comparative determination of blood iron has been made by the new colorimetric method and by Wolter's volumetric method (4) applied to the ash of 5 cc. of blood. The close agreement of the results obtained is shown in Table I. A pure specimen of methemoglobin of dog has also been analyzed by the new method. The iron obtained and that calculated agree closely as seen in Table II.

SUMMARY.

Another simple and rapid colorimetric method is described for the determination of iron in blood.

BIBLIOGRAPHY.

1. Wong, S. Y., *J. Biol. Chem.*, 1923, lv, 421.
2. Hufner, G., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1894, 130.
3. Jaquet, cited in Robertson, T. B., Principles of biochemistry for students of medicine, agriculture and related sciences, Philadelphia and New York, 1920, 350.
4. Wolter, O., *Biochem. Z.*, 1910, xxiv, 108.

A SIMPLE RESPIRATION APPARATUS FOR DETERMINATION OF OXYGEN AND CARBON DIOXIDE IN INDIRECT CALORIMETRY.

By J. F. McCLENDON, HILDING C. ANDERSON, F. R. STEGGERDA, CLAIRE CONKLIN, and MILDRED WHITAKER.

(From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.)

(Received for publication, March 8, 1928.)

Although many forms of respiration apparatus have been described by Benedict and Collins (1), Hagedorn (2), Knipping (3), Krogh (4), McClendon, Humphrey, and Loucks (5), and Roth (7), we had not found one quite suitable for the use of students in the general course in physiological chemistry. Since basal metabolism is of diagnostic importance and the general course is all that is required of medical students, further simplification of the method seemed desirable. The student is required to show skill in the volumetric measurement of liquids but the measurement of gas volumes necessitates temperature control and detection of leaks in a manner quite different. The apparatus described below is an improvement on that of McClendon and Van Slyke (6) (Fig. 1).

The principle of our method is the immersion of all the apparatus in a single water bath of such size that the heat transmitted to the air in the lungs and the heat generated in absorption of CO_2 do not change the temperature of the bath significantly. Furthermore, the spirometer domes when projecting from the water are enclosed in a box so insulated as not to vary from the temperature of the water to a significant degree. The apparatus is kept in a room with ordinary temperature regulation and without large window or outside wall area.

The essentials of the apparatus are shown in Fig. 2. The water bath is 48 inches high, 40 inches wide, and 20 inches thick, with a $10 \times 10 \times 48$ inch enlargement in front (for the soda-lime con-

tainer). In it are immersed two cylindrical celluloid (or copper) spirometer domes each 100 cm. high, of 35.68 cm. bore, 112.1 cm. internal circumference (112.4 cm. before bending), 0.0627 cm. (0.025 inches) thick, and of 100 liters capacity. The center of

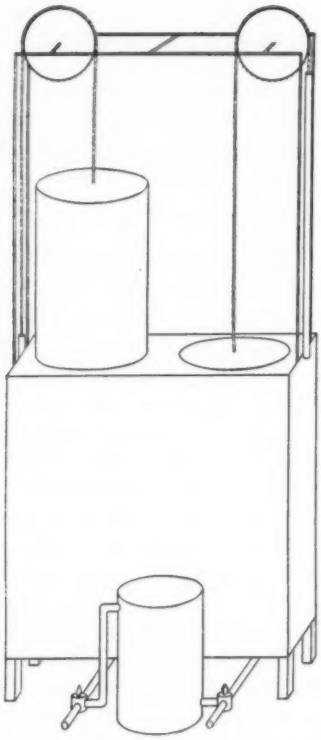

FIG. 1. The prototype of the respiration apparatus of McClendon and Van Slyke.

the top of each spirometer dome is connected to a $\frac{3}{16}$ inch roller bicycle chain (1 inch pitch) 150 cm. long, weighing 520 gm. (each cm. weighs 3.53 gm.), that passes over a 30 tooth bicycle sprocket wheel, 25 cm. in diameter, and is attached to a meter stick marked in mm., for indicating the volume, and backed with lead so

as to counterbalance the weight of the dome. A vernier attached to the water bath marks the point to be read on the scale of the meter stick.

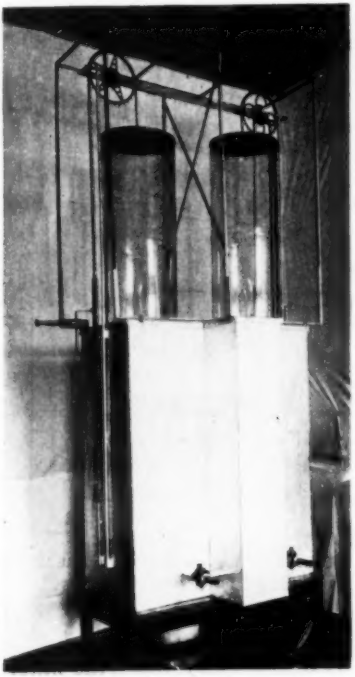

FIG. 2. The respiration apparatus with celluloid spirometer domes having meter scales attached and telescope in place at left for reading this scale. A frame over the apparatus shows the outline of the wall-board box that was applied later.

When the dome is immersed to the depth of 1 cm. in the water, owing to the thickness of its wall it displaces $0.0627 \times 112.4 \times 1 = 7.08$ cc. of water which weigh $7.08 \times 0.997 = 7.06$ gm., but the chain is reduced 3.53 gm. on the scale side and increased 3.53 gm. on the dome side and hence the changed setting of the

chain compensates for the buoyancy due to displacement of water. During experiments, one dome goes up as the other goes down and the general water level is not changed.

On the front of the water bath is the enlargement containing a can (4 inch bore, 48 inches high) with a wire gauze false bottom 8 cm. from the bottom and with the remainder filled with 5 kilos of soda-lime, and the top and bottom closed air-tight. The bottom of the soda-lime can is connected by means of a tube of 2.5 cm. bore to a 3-way valve on the right and the top by means of a similar tube (bent down to the base of the can) to a 3-way valve on the left.

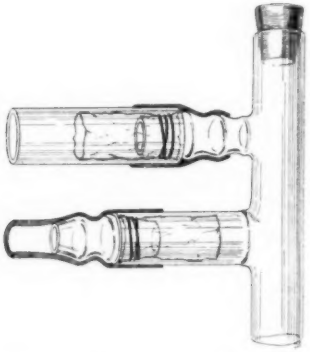

FIG. 3. Valve container. Valves made of Visking sausage casings in 1 inch glass tubing connected with rubber tubing.

Each 3-way valve is connected to a flattened tube of 1 × 10 cm. cross-section extending up through the water into the spirometer. The tube on the left contains a strip of cloth which is folded over into the water and acts as a wick to keep the air in the tube saturated with moisture. The bottoms of the flattened tubes are connected to the outside by means of U-tubes filled with water which allow water overflowing into the tubes to escape and not go into the patient's mouth.

A mouthpiece or mask is applied to the patient and is connected to the open tube of the valve container shown in Fig. 3. The two side arms of the valve container are fitted with 4 cm. lengths of Visking sausage casing (Visking Corporation, Union Stock Yards,

Chicago) that act as valves. One of these valves is connected by rubber tubing (1 inch bore) to the left 3-way valve and the other to the right. The valves are kept moist with glycerol.

In using the apparatus, we fill the left spirometer with fresh air, the CO_2 of which is absorbed by drawing it through the soda-lime, and its scale read; and the right spirometer is emptied (scale reading zero). A thermometer in the water is used to determine the temperature. The 3-way valves are turned so as to close off the soda-lime can and connect the spirometers with the mouthpiece. The mouthpiece and nose clip are attached to the patient (the stopper being removed from the valve apparatus). At the end of an expiration the stopper is inserted and a stop-watch started. Air passes from the left spirometer into the lungs of the patient and out into the right spirometer. When the left spirometer is nearly empty (in about 12 to 15 minutes) but at the end of an expiration, the stopper is removed, the stop-watch stopped, and the 3-way valves turned so as to cut off the spirometers. The scales of both spirometers are read and recorded. The 3-way valves are turned so as to cut off the mouthpiece but connect the spirometers through the soda-lime can. A 100 gm. weight is attached to the meter stick of the left spirometer which will slowly force the air from the right spirometer through the soda-lime into the left spirometer.

The difference in the volume of the content of the left spirometer at the start and after receiving the air through the soda-lime is the oxygen absorbed by the lungs (assuming no nitrogen to be absorbed). The difference in the volume of the content of the right spirometer and the final reading of the left spirometer is the CO_2 given out by the lungs. These gases are measured saturated with moisture at the temperature of the water bath and must be reduced to dryness, 0° and 760 mm. of Hg.

The accuracy of the method depends on the constancy of the temperature. The room temperature should be very close to that of the water bath. We have used celluloid as the poorly conducting material for the walls of the spirometers. These spirometers easily become warped. We have also used copper spirometers varnished with celluloid. We have dissolved celluloid in 40 per cent amyl acetate + 60 per cent acetone and found it satis-

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factory as a varnish or cement for uniting pieces of celluloid when applied in a dry atmosphere. We applied a number of coats of celluloid varnish to the copper spirometers in order to decrease heat conduction.

The most effective method of inhibiting heat transfer is the enclosure of the space occupied by the domes when they rise. This enclosure is best made of non-conducting wall-board (Insulite or Celotex) and coated inside with paraffin after having been made in the form of a box. The front of the box may be removed in order to get at the spirometer domes.

The accuracy of the method depends on the constancy of the temperature and the accuracy of the mechanical construction, since 1 mm. on the scale is equivalent to 100 cc., and 0.1 mm. may be read on the vernier. If the experiment is continued until the left spirometer is nearly empty, about 3 liters of O_2 will be absorbed and the reading of the scale will be within 1 per cent. It is necessary to have the bicycle wheels perfectly round. This can be tested by attaching a meter stick to the spirometer dome and reading it with a telescope shown in Fig. 1 at the same time that the regular scale is read. This is repeated for several levels of the spirometer dome. The right and left spirometers are checked against one another by connecting them through the 3-way valves before the soda-lime is introduced.

The calorimeter is essentially a Haldane gas analysis apparatus. It has, however, two disadvantages over the ordinary Haldane apparatus. First, it is of larger size and hence more difficult to keep all parts at the same temperature, and secondly, the portion of the scale that is used cannot be stretched out as in the (glass) Haldane apparatus. The chief advantage over the Haldane apparatus is the avoidance of the necessity of washing the air out of the connecting tubes (on account of their small volume in comparison to the size of the apparatus). After the soda-lime has been used a great number of times, it is necessary to verify complete absorption of CO_2 by passing the air again through the soda-lime but always measuring it in the left spirometer (because this has the wick for saturation with moisture). The soda-lime may be removed by opening the tube containing it at the bottom and removing the wire screen false bottom, allowing it to drop out. Detection of leaks is made by turning the 3-way valves as for

absorption of CO_2 and placing a 2 kilo weight on each dome. The reading of both scales is recorded and if found to decrease in 10 minutes the leak is localized by various settings of the valves. Minute leaks may be stopped with beeswax or a mixture of beeswax and vaseline.

The apparatus has been used to determine basal metabolism in men and women and the normal subjects show normal values. Alcohol checks were made by burning weighed quantities of absolute ethyl alcohol (99.5 per cent alcohol, free from aldehyde) in a Pyrex alcohol lamp inside a lamp-chimney with a condenser at the upper end. The lower part of the lamp-chimney was connected to the left spirometer and the upper part through the condenser to the right spirometer while the air was slowly forced from the left through the chimney into the right spirometer. The respiratory quotient in the alcohol checks varied from 0.652 to 0.675.

The following is an example of an alcohol check. In burning 6.7 gm. of alcohol, 9.78 liters of O_2 (corrected) were used and 6.47 liters of CO_2 (corrected) were produced, giving a respiratory quotient of 0.662. The theoretical O_2 is 9.79 liters and theoretical R.Q. is 0.666. The low value of the R.Q. may be partly due to failure to wash out the CO_2 from the connecting tubes and about ½ inch of space at the top of the right spirometer when it reads zero. This CO_2 could be washed out, but as we do not do so in the routine determinations of respiration of man we did not do so in the alcohol checks.

The alcohol checks are more accurate than measurements of respiration due to the variable capacity of the "collapsed" lungs. The flip of the valves is used to determine the regularity of respiration.

BIBLIOGRAPHY.

1. Benedict, F. G., and Collins, W. E., A clinical apparatus for measuring basal metabolism, *Boston Med. and Surg. J.*, 1920, clxxxiii, 449.
2. Hagedorn, H. C., Apparatus for the graphic recording of oxygen consumption and carbon dioxide output, especially adapted for clinical work, *Biochem. J.*, 1924, xviii, 1301.

3. Knipping, H. W., A simple apparatus for exact gas metabolism determination, *Z. ges. exp. Med.*, 1924, xli, 363.
4. Krogh, A., A respiration apparatus for the clinical determination of energy metabolism of man, *Wien. klin. Woch.*, 1922, xxxv, 290; *Compt. rend. Soc. biol.*, 1922, lxxxvii, 458; *Boston Med. and Surg. J.*, 1923, clxxxix, 313; *Ugesk. Læger*, 1922, lxxxiv, 525.
5. McClendon, J. F., Humphrey, G. J., and Loucks, M. M., A portable calorimeter for the determination of both oxygen and carbon dioxide, *J. Biol. Chem.*, 1926, lxix, 513.
6. McClendon, J. F., and Van Slyke, C. J., An indirect calorimeter for the determination of O_2 and CO_2, *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 925.
7. Roth, P., Modifications of apparatus and improved technic adaptable to the Benedict type of respiration apparatus. III. Graphic method for the estimation of the metabolic rate, *Boston Med. and Surg. J.*, 1922, clxxxvi, 491.

A NEW BLOOD SUGAR METHOD.

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The purpose of the investigation described in this paper was to develop a method for the determination of sugar in 0.1 cc. of blood. Heretofore I have not been much interested in so called micro methods of blood analysis, because one usually wants to determine more than one blood constituent, and whenever that is the case it is more practical to use larger quantities of blood and make the several determinations on one filtrate. The sugar problem is, however, so distinct from the nitrogen problem that it would seem to merit separate consideration.

In this method I have abandoned the use of copper solutions. The sugar is oxidized with alkaline potassium ferricyanide, and the ferrocyanide produced is measured colorimetrically as Prussian blue. The color obtained from 0.04 mg. of glucose in a 25 cc. tube is quite as deep as the color obtained from 0.2 mg. of sugar in the Folin-Wu method. Two separate determinations, therefore, can be made on the extract from 0.1 cc. of normal blood, and several determinations can be made when the extract is obtained from diabetic bloods with high sugar contents.

Reagents.

1. Dilute Tungstic Acid Solution.—Transfer 20 cc. of 10 per cent sodium tungstate solution to a volumetric liter flask. Dilute to a volume of about 800 cc. Then add with shaking 20 cc. of $\frac{2}{3}$ N sulfuric acid and dilute to volume. Add a little toluene to prevent the growth of moulds.

2. Potassium Ferricyanide Solution.—Dissolve 1 gm. of C.P. potassium ferricyanide in distilled water and dilute to a volume of 500 cc. The major part of this solution should be kept in the dark.

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3. Sodium Cyanide-Carbonate Solution.—Weigh out 2 to 4 gm. of Merck's sodium cyanide and dissolve in enough distilled water to make approximately a 1 per cent solution. Transfer 8 gm. of anhydrous sodium carbonate to a 500 cc. volumetric flask and dissolve it by means of about 100 cc. of distilled water. With a cylinder add 150 cc. of the 1 per cent cyanide solution. Dilute the cyanide-carbonate mixture to volume, 500 cc., and mix. Discard the remainder of the cyanide solution.

4. Ferric Iron Solution.—Transfer 30 gm. of gum arabic to a liter Florence flask and add 600 cc. of distilled water. Heat on the water bath, with shaking, until the gum has all dissolved. Transfer 5 gm. of ferric sulfate, $Fe_2(SO_4)_3 \cdot 9\ H_2O$, to another smaller flask. Add 75 cc. of 85 per cent phosphoric acid and 100 cc. of water. Heat until the sulfate has dissolved. Cool both solutions and transfer them to a volumetric liter flask. Dilute to volume by gentle inversions. Vigorous shaking is undesirable, because the gum arabic tends to produce rather permanent foam. The solution may be filtered, but the filtration is slow and is scarcely necessary.

5. Standard Stock Solution of Glucose.—Dissolve 2 gm. of benzoic acid in about 500 cc. of hot distilled water in a liter Florence flask. Weigh out 2 gm. of C.P. (Bureau of Standards) glucose. Rinse the glucose by means of the warm benzoic acid solution into a volumetric liter flask. Add water to about 900 cc., cool to room temperature, dilute to volume, mix, and transfer to a clean glass-stoppered bottle. The solution contains 2 mg. of glucose per cc.

From this solution one obtains the dilute working standard, containing 0.01 mg. of glucose per cc., by diluting 200 times.

Transfer 0.5 gm. of benzoic acid to a 2 liter volumetric flask and add about 1500 cc. of distilled water. Add 10 cc. of the stock solution (5), and shake occasionally until the benzoic acid has dissolved. Dilute to volume, mix, transfer to a glass-stoppered bottle, and add a little toluene. It is best not to be constantly opening and contaminating this solution. Use only a part of it, in a separate container.

A little supplementary discussion concerning two of the reagents employed may prove helpful.

A.—The ferric iron solution is perhaps unnecessarily strong in

gum arabic, but the Prussian blue which it yields when added to ferrocyanide solutions is very insoluble, and the uniform colloidal suspensions first obtained are soon converted into precipitates except for the presence of the protective colloid. Even with the 3 per cent gum arabic I find that the colorimetric plungers are apt to get coated with a thin blue film in the course of a long series of determinations. This film is instantly destroyed by immersing the plungers in a very dilute solution of sodium hydroxide. It may be remarked in passing that a small beaker of water is better than the colorimeter cups for rinsing the colorimeter plungers after each determination.

B.—None of the available C.P. grades of potassium ferricyanide is completely free from ferrocyanide, but the traces of ferrocyanide present are so small in Merck's, Baker and Adamson's, and Baker's C.P. ferricyanides that these brands can be used for routine determinations without any preliminary purification.

No ferricyanide should be used, however, without first testing for ferrocyanide. This test is made as follows: Transfer 2 cc. of a 1 per cent solution of the salt to a clean test-tube, add 3 cc. of water and 1 cc. of ferric iron solution (4); the amount of blue color observed at the end of 5 minutes will show whether the product is reasonably pure. Practically every trace of ferrocyanide can be removed by a single recrystallization according to the process described below.

Purification of Potassium Ferricyanide.—Transfer 100 gm. of C.P. potassium ferricyanide and 400 cc. of water to a liter beaker. Set the beaker in warm water (50°) and stir until solution is obtained. Simultaneously with this stirring, wash a 24 cm. filter on a suitable funnel with distilled water. Filter the solution through the washed filter into a flask; cover the mouth of the flask with a beaker, and cool the filtrate under running water. Transfer 600 cc. of alcohol to a flask and to it add about 0.1 cc. of bromine, shake a moment; transfer the cold ferricyanide solution to a 2 liter beaker and add, with stirring, the brominated alcohol. The ferricyanide comes down at once. Filter immediately through a well fitting hardened filter paper on a Buchner funnel (10 cm. diameter). Wash the (very fine) precipitate on the funnel with 150 cc. of alcohol to which have been added 2 or 3 drops of bromine; then wash with 100 cc. of ether also charged with bromine to a good straw-yellow color, and wash finally once with 25 cc. of ether containing no bromine. The last ether should be removed as thoroughly as possible by strong suction. Blow the precipitate on to a large watch-glass or plate and dry at about 50°. Yield 80 gm.

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The secret of the success of this recrystallization is, of course, the presence of bromine. The same process without bromine would yield a product materially worse than the starting product. As the bromine is gradually used up by the alcohol and ether it is desirable to work rather rapidly. The whole process, aside from the final drying, should be finished in about 2 hours.

Preparation of the Protein-Free Blood Filtrate.

One would ordinarily expect that the preparation of an adequate amount of blood filtrate from 0.1 cc. of blood would be more difficult than when working with larger quantities of blood. But aside from the one point of measuring 0.1 cc. of blood, the preparation of the filtrate is even simpler than is the preparation of the usual filtrates. This is so for two reasons. First, because one does not need to measure out separately, tungstate, acid, and water; the dilute tungstic acid solution (1) yields perfect precipitation and practically at once. Second, in this case the centrifuge alone gives water-clear supernatant solutions; this saves time and material and incidentally eliminates the danger of contaminating the filtrate with reducing materials from filter paper.

The preparation of the usual, 1:10, Folin-Wu blood filtrates from small amounts of blood is often beset with difficulties. The tubes in which the bloods are collected usually contain enough anticoagulant (oxalate, fluoride, or citrate) for at least 10 cc. of blood. If then only 1 or 2 cc. of blood are actually collected the result is a relatively enormous excess of anticoagulant. In the hands of technicians these conditions are very apt to yield more or less colored or very acid filtrates, and erroneous figures. The disturbing effects of too much anticoagulant are less prominent, if not entirely eliminated, when the precipitation is made under the new conditions described above. Yet in actual work with finger blood one uses no anticoagulant at all.

The method described here should be particularly useful for work on small animals, but its widest use should be in its application to human subjects. Even among physicians there are still many who dislike, for lack of experience, to draw blood from a vein, and non-medical laboratory workers often hesitate for fear of possible legal consequences. Scarcely any one will hesitate about pricking a little hole through the skin deep enough to get

2 or 3 drops of peripheral blood. No skill is required, since an ordinary spring lancet will do it automatically.

As a novice in the use of such blood I have felt the lack of definite information as to how it should be taken. When blood is taken from the lobe of the ear, a finger tip, or near the root of a finger nail, it would seem more or less inevitable that the first step would be and has been to collect a few drops in some receptacle, just as is usually the case when blood is taken from a vein.

While such a process can be made fairly satisfactory and in some cases may be unavoidable, it is open to the objection that considerable error may occur, because of evaporation. Since an excess of blood must be collected, it also defeats, in a measure, the primary aim of a micro method. While studying this problem I found that very practical 0.1 cc. pipettes can be made, the filling of which requires neither the force of gravity nor suction. In glass tubes having an internal diameter of 1.0 to 1.7 mm., water will rise by capillary attraction alone to a vertical height of 1.5 to 2 cm. 0.1 cc. will fill such tubes to a length of from 5 to 8.0 cm. (from the tip). Pipettes made from such tubing can thus be filled automatically to the 0.1 cc. mark just as the capillary 0.01 cc. blood count pipettes are filled; in fact, much better, because the speed with which the first named fill is slower, and can be regulated by varying the upward angle at which they are held.

From the 0.1 cc. and 0.2 cc. pipettes with attached rubber tubing and mouthpiece, already listed in catalogues, one may infer that some attempts have been made to collect blood directly from the bleeding finger or ear by means of such pipettes, but any one who will try to do so will find that the failures are altogether too numerous because of sucked in air bubbles. The automatic pipettes described here do not collect air bubbles. The automatic pipettes are made to *contain* (not to deliver) 0.1 cc.

They must be accurate, of course, and should be calibrated by means of mercury (0.1 cc. of mercury weighs 1.355 gm.). To fill such a pipette exactly to the 0.1 cc. mark with mercury, collect an excess of mercury by suction, then hold the pipette in a nearly horizontal position over a beaker and gently tap and scrape the tip against a watch-glass. The excess of mercury can thus be removed in the form of exceedingly small globules, until the upper level corresponds exactly with the ring representing the 0.1 cc. mark.

The analyses reported in this paper were made with the help of home-made pipettes, but very good "micro blood pipettes" can now be obtained from Eimer and Amend.

One quite essential point in connection with these pipettes is that they must be clean—free from fat films. Unless they are clean they will not fill spontaneously. After they have been used, they should be immersed, therefore, in cleaning fluid, at least for a few minutes, before being rinsed and dried.

The accurate collection of as little as 0.1 cc. of blood has been one of the important obstacles to the use of so called micro methods, but I am now positive that no significant error need be made in the collection of 0.1 cc., by means of the pipette described above.

The collection is made as follows: Wash the finger with soap and hot water. Apply the lancet firmly and release the spring. If the cut is deep enough sufficient blood will come out freely; if it comes only very slowly, a little firm milking movement or pressure applied to the finger will bring it out. It is not at all necessary or even desirable that 0.1 cc. should have accumulated before one applies the pipette. Collect that which has come, and press out some more, and repeat until the pipette is filled to the mark. If the pipette is clean, it will take up the blood so neatly that scarcely one single extra drop is necessary for the accurate collection of 0.1 cc. Collect the blood to the 0.1 cc. mark, or a little above; wipe off the tip with a piece of soft filter paper or cloth; adjust the level of the blood to the 0.1 cc. mark by means of capillary suction from the paper (or cloth) and transfer at once to a 15 cc. centrifuge tube containing 10 cc. of dilute tungstic acid solution. Rinse the pipette with the contents of the centrifuge tube.

A piece of fine linen handkerchief is better than filter paper for adjusting the blood to the 0.1 cc. mark.

A still better method is to use a clean glass surface such as the side of a test-tube and to depend partly on the force of gravity:

Wipe off the outside blood with a filter paper, then slowly draw the pipette, as though it were a fountain pen, along the glass surface until the correct level is attained. If accidentally a little too much blood has escaped, it remains on the glass and can be drawn into the pipette again by moving the pipette toward a more horizontal position. A second wiping of the pipette can be done after the adjustment is finished by first slanting the pipette so that the blood moves away from the tip. It is doubtful, however, whether the second wiping is called for.

In this process no oxalate or other anticoagulant is used, for there is ample time for the collection and transfer of the blood before clotting occurs.

Stir the mixture in the centrifuge tube thoroughly, preferably at once, with a rather fine glass rod or with the pipette. Remove the rod and centrifuge for 3 to 5 minutes. Fully 9 cc. of perfectly water-clear solution are obtained, which in normal cases will contain from 0.007 to 0.014 mg. of glucose per cc.

Determination.—Transfer 4 cc. of the blood extract to a test-tube graduated at 25 cc., and transfer 4 cc. of the standard glucose to another graduated test-tube. By means of burettes, or 5 cc. Folin-Wu blood pipettes, add 1 cc. of the 0.2 per cent potassium ferricyanide solution (2) and 1 cc. of cyanide-carbonate solution (3) to each test-tube. Heat both tubes in a beaker of boiling water for 8 minutes. Cool, and add (with a 10 cc. blood pipette) 3 cc. of the acid ferric iron solution (4) to each tube. The iron solution should be added down the side of the tube to prevent foaming. Mix by a little gentle shaking, let stand for 5 minutes, then dilute to volume, mix, and make the color comparison, not omitting first to read the standard against itself.

If the standard, 0.04 mg., is set at 20 mm., then $\frac{20}{R} \times 100$ gives the sugar of the blood in mg. per cent (mg. per 100 cc.).

This calculation is subject to a theoretical, but negligible, error, in that no account is taken of the water added in the form of blood.

If R, the reading of the unknown, is over 30 or under 13, it is perhaps best to repeat the determination, 2 cc. of the standard, or 2 cc. of the unknown, plus 2 cc. of water being used. When purified ferricyanide is used, one can as a matter of fact make perfect color comparison even when the standard or the unknown is twice as strong as the other, by first making a suitable adjustment of the yellow color due to surplus ferricyanide. When the colors of the standard and the unknown are seen (before the dilution) to be very far apart, include in the dilution the addition of up to 1 cc. of ferricyanide solution to the tube whose contents are much more blue than the other. By this simple device, which takes only a moment and requires very little accuracy or judgment, one can get practically perfect figures for 0.02 mg. and for 0.08 mg. of glucose when read against the 0.04 mg. standard.

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When working with diabetic blood filtrates it is, of course, essential to make sure that an excess of sugar is not present. Some yellow color from unreduced ferricyanide must be present when the tubes are removed from the water bath.

One word of caution may be advisable. It would be a trifle simpler first to mix equal volumes of reagents (2) and (3), the alkali and ferricyanide, and then add 2 cc., instead of adding 1 cc. of each, as described. Such a simplification would be sure, however, to lead to many less accurate results. Even very slightly alkaline solutions of potassium ferricyanide gradually yield ferrocyanide especially when exposed to light, even diffuse daylight. This change is particularly rapid when the alkali contains sodium cyanide, and in this case, curiously enough, the autoreduction to ferrocyanide takes place more rapidly at room temperatures than in boiling water. It is, therefore, clearly safer and more correct to add the alkali separately and only just before one is ready to put the tubes in the boiling water.

It will be noted that the Folin-Wu blood sugar tubes, so essential in the copper methods, are not prescribed in this new method. They are not prescribed because in this case there is no danger of reoxidation.

Inasmuch as the new method described in this paper is quite different from any other method, it has seemed desirable to show by a series of analyses how the figures which it yields compare with the figures obtained by the Folin-Wu method.

In this work I have enjoyed the very competent assistance of H. I. Coombs, who also has made some accurate 0.1 cc. pipettes for me.

Since the new method presumably will be used mostly with capillary blood, while the Folin-Wu method is used almost entirely with venous blood, a comparison between the two methods must include, first, a comparison based on the use of the same sample of (venous) blood or blood filtrate, and, second, a comparison based on venous and capillary blood. Comparisons of this kind are shown in Tables I and II.

In order to determine the true difference in sugar content of venous and capillary blood one must, of course, use the same method on both. A comprehensive study of this sort does not come within the scope of this paper. But our determinations

have incidentally supplied us with a few figures obtained by the new method on venous and capillary blood. These are given in Table III.

TABLE I.

Showing That on the Same Sample of Blood (or Blood Filtrate) the New Method Gives Unmistakably Lower Values than the Folin-Wu Method.

Sample No.	Sugar in mg. per cent.		
	Folin-Wu.	New method.	Difference.
1	308	280	28
2	288	272	16
3	286*	232*	54
4	294	285	9
5	230	218	12
6	220	202	18
7	196	186	10
8	164	148	16
9	150	138	12
10	143	138	5
11	122	113	9
12	116	99	17
13	115	99	16
14	108	95	13
15	105	91	14
16	93	88	5
17	110†	98†	12
18	180†	168†	12

* This determination was verified by repetitions.

† Nephritic blood high in non-protein nitrogen.

TABLE II.

Indicating That the New Method with Capillary Blood Will Usually Yield Somewhat Lower Values than Are Obtained on Venous Blood by the Folin-Wu Method.

Subject.	Sugar in mg. per cent.			
	Venous blood. Folin-Wu method.	Finger blood. New method.	Difference.	Remarks.
Ci-i.	115	106	9	6 hrs. after meal.
Br-d.	105	94	11	6 " " "
Br-n.	122	113	9	½ hr. " "
Wo-n.	93	94	−1	17 hrs. " "
Bu-n.	108	95	13	3 " " "
Se-l.	108	99	9	1½ " " "

TABLE III.

***Simultaneous** Sugar Values Obtained on Venous and Capillary Blood of Normal Persons (Students), with New Method.*

Subject.	Sugar in mg. per cent.			
	Venous blood.	Finger blood.	Difference.	Remarks.
Ci-i.	99	106	7	6 hrs. after meal.
Br-d.	91	94	3	6 " " "
Br-n.	102	113	11	½ hr. " "
Wo-n.	93	88	−5	17 hrs. " "
Bu-n.	108	95	13	3 " " "

ACCELERATION OF GROWTH RATES BY DIETARY MODIFICATIONS.*

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Recent investigations have indicated that "normal" growth may fall far short of optimal or maximum growth. Thus Osborne and Mendel (1) devised rations that brought about an accelerated growth rate of rats, far more rapid than any previously recorded, and so far as we are aware the first successful attempt to induce "supernormal" growth by dietetic measures. These investigators prepared rations that, so far as present knowledge goes, supply all nutrients in liberal quantities, but the accelerated growth rate was not definitely associated with any recognized constituent.

Our interest in the possibility of greatly improving growth rates, by adjustments of the ration, began during our studies of the nutritional requirements of the chick. It was a common experience that within the same group some individuals would respond to the ration much more satisfactorily than others, and we gradually became convinced that the high degree of variability was due to unsuspected dietary inadequacies. We then attempted to devise a ration that would permit a group of chicks to grow at a more uniform rate, and the one finally adopted has been quite satisfactory. The mortality is low, the variability is reduced, and the average rate of growth is much more rapid than we had previously obtained. The composition of the ration, our Ration 634, is given in Table I. Our method of caring for baby chicks is described in some detail in a previous publication (2) from this laboratory.

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Before proceeding with a description of our own work, it might be well to mention briefly some of the data available on the growth rate of the White Leghorn chick. The most rapid growth hitherto recorded that has come to our attention, at least for the first 12 weeks after hatching, was reported from the Kentucky Agricultural Experiment Station (3). In this paper the growth of two groups is described, one of which was reared artificially in a brooder, while the other group was reared with hens, under more natural condition. The mortality was lower in this latter group, and the growth rate higher.

During our earlier studies, concerned primarily with the effectiveness of Ration 634, we used thirty-seven individuals, divided in three groups. In one group of four, and another of seven, there were no mortalities, but in another group of twenty-six there were four deaths within the first 4 weeks, though we are not certain that any of the mortalities could be attributed to the ration. One death was due to accidental injury in the brooder, one to an affection of the lungs, one to anal rupture, and one to intestinal obstruction. Regardless of the cause of the deaths, however, the total mortality up to 12 weeks was 11 per cent. Subsequently the chicks were transferred to less favorable quarters, and there were two more deaths, which we attributed to close confinement during the high summer temperatures. These two individuals are not included in our average weights, however.

During more recent investigations, not described in detail in this paper we reared fifteen chicks on Ration 634, with no mortalities. In a group of seventeen, started on a modification of this diet, however, there were two mortalities. The solid portion of the ration was the same, but instead of water the chicks were given skim milk to drink, kept before them constantly. We estimate then that of the original 69 chicks, the mortalities amounted to six, or 9 per cent.

Our earlier observations are summarized in Fig. 1. We are not including any measure of the degree of individual variability. However, of the thirty-one individuals included in Fig. 1, only one individual, a male, was below the normal weight, as described in the paper from the Kentucky station.

It would be desirable to know to what constituents of Ration 634 are to be ascribed its peculiar nutritional value, assuming that

it really has some unique properties. In our first attempts to secure a ration that is invariably satisfactory for the growth of chicks, we used a ration described by Slonaker (4), our Ration 400. The results with this diet were unsatisfactory, however, as some

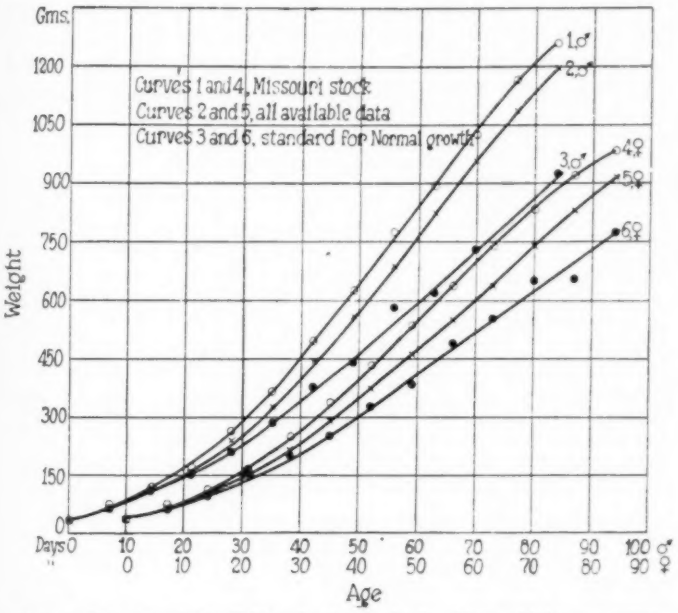

FIG. 1. Growth of the White Leghorn chick. Four chicks are represented in Curve 1, three in Curve 4, seventeen in Curve 2, and fourteen in Curve 5. The chicks described in Curves 1 and 4 were obtained from the University poultry flock. Curves 2 and 5 include these seven, and in addition twenty-four more obtained from a commercial hatchery. The standard of normal growth is the more rapid of the two reported by Buckner, Wilkins, and Kastle.

groups grew well, while others did very poorly. It seemed that the ration contained too low a proportion of some essential constituent, or constituents, so it was modified to form Ration 634, which seemed entirely satisfactory for our purpose. We have made, therefore, some preliminary observations that may indi-

cate the deficiencies of Ration 400, when used as a diet for baby chicks. The rations used are given in Table I.

We have not attempted an exact comparison of these four rations, but our data, even though limited, have led us to believe that neither Ration 696 nor 697 is much superior to Ration 400, and that all are definitely inferior to Ration 634. We have tabulated the weights of comparable groups in Table II.

It would be interesting to know more precisely why Ration 634 is superior to No. 400 for our purpose, but we have not attempted

TABLE I.

Composition of Rations.

Constituents.	Ration 400.	Ration 696.	Ration 697.	Ration 634.
	per cent	*per cent*	*per cent*	*per cent*
Whole wheat	65.5	57.6	63.5	55.6
" milk powder	9.7	8.5	9.4	8.2
Commercial casein	14.5	12.8	14.0	12.3
Alfalfa meal	2.9	2.6	2.8	2.5
Butter fat	4.9	4.3	4.8	4.2
Sodium chloride	1.0	0.9	1.0	0.9
Calcium carbonate	1.5	1.3	1.5	1.3
Cod liver oil			3.0	3.0
Dried yeast		12.0		12.0

TABLE II.

Growth of the Chick on Modifications of Ration 400.

Ration No.	400		696		697		634	
No. of individuals	5♂	5♀	3♂	5♀	3♂	3♀	5♂	7♀
Average weight at 8 wks., *gm.*	412	346	458	402	440	347	720	559

as yet to obtain definite evidence. Our original hypothesis, however, was that Ration 400 does not supply the chick with enough of either vitamin B or vitamin D for maximum growth.

We have also attempted to secure supernormal growth of the turkey, though we are uncertain as to the degree of success we have obtained, because of the lack of standards. The turks were supplied with Ration 634, and skim milk was kept before them constantly, water being entirely withdrawn. Until they were 8 weeks of age they were confined to small pens in the laboratory, but they

were then gradually transferred to outdoor pens[1] and allowed considerable range. Our observations are presented graphically in Fig. 2.

Standards for comparison are fragmentary but for our purpose the most useful data are those published by Brooks (5), who reported the weights of Bourbon Red turkeys at various ages. Since these were presumably reared under the most favorable conditions modern practice can supply, we believe a comparison of

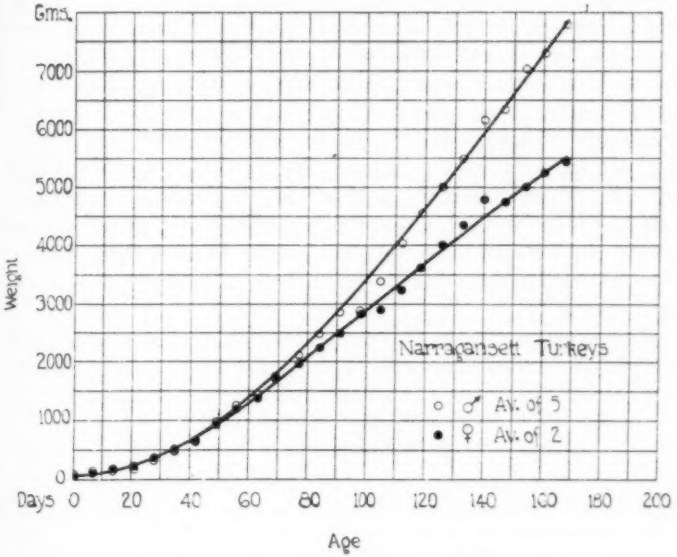

FIG. 2. Growth of the Narragansett turkey.

our data with his should give some indication as to whether or not the ration we have employed will support growth of turkeys at a more rapid rate than has been previously attained. Brooks does not report the sexes separately, but he states that in 1923, twenty-eight turkeys had an average weight of 11.07 pounds at 24 weeks

[1] We are greatly indebted to Dr. A. J. Durant of the Department of Veterinary Medicine, who took charge of the turks at this time, and who kindly furnished us with a record of their weights.

of age. At that age the average weight for our males was 17.11 pounds, and for the females 11.88 pounds. In fact the lightest of our turkeys, a female, was heavier than the average reported by Brooks. Possibly we should add that Bourbon Red and Narragansett turkeys are about the same size, as indicated by breeders standards (6). According to these at 12 months the standard weight of a male is 20 pounds, and the standard weight of a female is 14 pounds.

We believe the point worth mentioning that though Ration 634 is superior to some others that have been used, we are not certain as yet that the rapid growth rate observed is due to the ration alone. Our feeding trials with chicks were carried out under strictly laboratory conditions, and so they were confined constantly to small pens. Ordinarily that would be regarded as a handicap, but it may be that confinement is conducive to rapid growth. Furthermore the quarters were thoroughly disinfected after each feeding trial, and so the growth rate may have been due merely to freedom from disease. Other possible explanations may be found in the avoidance of exposure to cold or wet weather, or to drafts.

We are attempting to devise a practical poultry ration that will support supernormal growth, but it remains to be seen whether such a ration is of any value under commercial conditions.

BIBLIOGRAPHY.

1. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1926, lxix, 661.
2. Hogan, A. G., Guerrant, N. B., and Kempster, H. L., *J. Biol. Chem.*, 1925, lxiv, 113.
3. Buckner, G. D., Wilkins, R. H., and Kastle, J. H., *Am. J. Physiol.*, 1918, xlvii, 393.
4. Slonaker, J. R., *Am. J. Physiol.*, 1925, lxxi, 302.
5. Brooks, F. D., *Poultry Sc.*, 1927, vi, 233.
6. American Poultry Association, American standard of perfection, Mansfield, Ohio, 1926, 422, 424.

THE VALUE OF IRON SALTS IN COUNTERACTING THE TOXIC EFFECTS OF GOSSYPOL.*

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The fatal results which often follow the prolonged feeding of large amounts of cottonseed meal to many animals are well known. Many attempts have been made to avoid such results and some have met with considerable success. Several years ago Withers and Brewster (1) reported successful attempts to avert this so called cottonseed meal toxicity in rabbits by administering iron salts to the animals on cottonseed meal diets. These investigators were led to employ iron as an antidote on the assumption that the toxic principle of the meal was a constituent of the protein molecule which acted upon the iron of the blood. Later researches by Withers and Carruth (2) showed the toxic substance to be gossypol. In the study first mentioned, a number of animals was given access to cottonseed meal diets which furnished 15 gm. of cottonseed meal per animal, per day. Another group of animals was given the same amount of meal, with the addition of 0.3 gm. of citrate of iron and ammonia. As a result, the average span of life for the animals not receiving iron was 13 days, but the animals receiving the iron in their diet were alive and normal after 41 days.

The investigation was continued by Withers and Carruth (3), who employed copperas and ferric chloride in two different amounts to overcome cottonseed meal poisoning in young pigs. Their results show that the iron had a decidedly beneficial effect, since deaths were either postponed or averted, and the animals made better gains.

The amount of soluble iron used in these feeding experiments was in the case of the rabbit feeding 0.4 gm. per 100 gm. of meal, and in the case of the pig feeding 0.4 gm. in one instance, and twice this amount in another instance, per 100 gm. of meal. Since the only source of iron, other than that purposely added, was confined to the small amount present in the meal and corn which made up the diets, the investigators were of the opinion

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that the beneficial effects of the added iron were "due to the formation of an insoluble iron salt of gossypol or one of its derivatives together with the catalytic acceleration of the oxidation of gossypol and perhaps a tonic action of iron on the system."

McGowan and Crichton (4), who also studied the effect of iron on the well being of pigs on cottonseed meal diets, ascribed the deleterious effects of the meal to a deficiency of iron, but many other experiments have quite conclusively proved that although injury may sometimes result from a deficiency of iron, excellent results can usually be obtained if the gossypol is destroyed before feeding the meal. Quite recently a number of studies dealing with the value of different forms of iron for correcting nutritional disorders has been reported, but there seems to be no direct connection between these studies and the one reported here.

EXPERIMENTAL.

This investigation had for its original purpose to determine whether the addition of 0.2 per cent ferric oxide (Fe_2O_3) to rations made up with cotton seeds for digestibility studies (5), would have a retarding effect on the physiological action of the gossypol. At the same time a much broader study dealing with certain phases of cottonseed meal feeding was being conducted in cooperation with the dairy department, and this study called for more definite information on the nutritive value of iron in cottonseed meal feeding. The scope of the project was therefore widened to include such information as could be obtained and appeared to be necessary for the proper procedure of the work. Albino rats, selected from a sturdy stock reared in this laboratory especially for this study, were used as experimental animals. They were kept under close observation in galvanized screen cages, and weighed every 10 days.

A quantity of cotton seeds sufficient to last throughout the experiment was delinted with sulfuric acid and analyzed for its gossypol content. It has been previously demonstrated (6) that the toxicity of cotton seeds is due to gossypol alone and is directly related to the amount of this substance present. It was first planned to extract the gossypol from the seeds and add to aliquot portions of the extract varying amounts of the iron salts. The extracts were then to be added to a basal diet for the rats. Since iron reacts almost immediately with a solution of gossypol in the test-tube, this procedure was abandoned on the grounds that it did not necessarily represent the reactions which might take

place in the digestive tract. Instead, the cotton seeds were used in diets in such amounts that they constituted either 10 or 20 per cent of the diet, and the iron salts were added in a finely powdered form. All the feeds were ground sufficiently fine to prevent a separation of the constituents by the animals. For controls by which comparisons might be made between the animals receiving the experimental diets and those subsisting on similar but non-toxic ones, a group of animals was given access to the same basal diet made up with autoclaved cotton seeds without the addition of iron salts. Autoclaving destroys the gossypol and leaves the seeds non-toxic (7). The composition of these diets was as follows:

Cotton seeds	20	10
Wheat	75	85
Cod liver oil	3	3
NaCl	1	1
$CaCO_3$	1	1
Iron salts.		

The experimental diets were prepared in small amounts and the cod liver oil added to a 10 day supply. The iron salts used in the first part of this study were ferric oxide (Fe_2O_3) and ferric citrate. The diets were prepared separately for each set of experiments in such a manner that only the percentage of wheat was varied slightly by the addition of different amounts of iron salts. The ratio of cotton seeds to iron was in every case, therefore, as 20 or 10 is to x, with x representing the different amounts of iron as calculated from the salt used. Several of these diets were analyzed for their total iron content, but the amount found was in such close agreement with the theoretical amount added that this step was not included in the general procedure. Special precautions were taken to avoid the scattering and separation of the food by the animals, by the use of feed containers which largely prevented scattering, and by restricting the daily allowance to amounts only slightly more than the average daily consumption. All of the animals, except those receiving the raw cotton seeds without iron, ate readily and showed no distaste for the food.

The results of the first part of this feeding work are shown in Table I. Unless otherwise stated, the growth records presented throughout this paper are the averages made by an equal number of males and females.

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RESULTS AND DISCUSSION.

A comparison of the results presented in Table I points to some relationship existing between the proportion of cotton seeds in the diet and the amount of soluble iron required to offset or delay their toxic effects. The toxic nature of the seeds is shown by the fatal results obtained when the seeds in the absence of added iron made up as much as 20 per cent of the diet, whereas the excellent results obtained when the autoclaved seeds were used in similar amounts verify the results reported in previous studies (7). The ferric oxide used in the two different amounts in combination with the

TABLE I.

Animals on Diets Containing Iron and 20 Per Cent Cotton Seeds.

Iron salt used.			Growth record.						
Ferric oxide.	Ferric citrate.	Iron per 100 gm. of cotton seeds.	Duration of experiment.	No. of animals.	Average initial weight.	Average gain.	Average loss.	Average daily gain.	Died.
per cent	*per cent*	*gm.*	*days*		*gm.*	*gm.*	*gm.*	*gm.*	
			30	4	80		15		2
(Autoclaved seeds.)			76	4	75	110		1.5	
0.2		0.7	30	4	86		13		
0.5		1.7	30	4	95		15		2
	0.3	0.3	76	4	94	38		0.5	
	0.6	0.7	76	4	115	60		0.8	
	1.5	1.7	76	4	94	66		0.9	
	1.5	1.7	76	4	49	95		1.2	
	3.0	3.4	76	4	89	24		0.3	

cotton seeds did not aid in overcoming the toxicity. The ferric citrate, when added to the extent of 1.5 per cent of the diet, allowed the animals to make the best gains, although their growth was still below normal and when this amount of iron was increased or decreased even smaller gains were made. No doubt the iron was itself a contributing toxic factor in the larger amounts, whereas in smaller amounts the toxic action of the gossypol predominated.

Since a diet composed of 20 per cent raw cotton seeds proved to be so extremely toxic, as a result of the high gossypol content, it seemed proper to reduce this amount to a level at which the animals would be able to make something like normal growth. If

the relation between iron and gossypol were a quantitative one, then it might be predicted that the level of iron might be lowered proportionally and results similar to those above obtained. Such proved to be true, as shown in Table II.

The analysis of the cotton seeds showed that they contained 0.503 per cent gossypol, and from the foregoing results it might be reasoned that nearly 2 gm. of iron were required for its complete destruction or precipitation in the digestive tract as an insoluble compound. Certainly some such situation as this must exist since

TABLE II.

Animals on Diets Containing Iron and 10 Per Cent Cotton Seeds.

Iron salt used.			Growth record.					
Ferric oxide.	Ferric citrate.	Iron per 100 gm. of cotton seeds.	Duration of experiment.	No. of animals.	Average initial weight.	Average gain.	Average daily gain.	Died.
per cent	*per cent*	*gm.*	*days*		*gm.*	*gm.*	*gm.*	
			76	4	72	63	0.8	
(Autoclaved seeds.)			76	4	65	101	1.3	
0.2		1.4	76	4	94	57	0.7	
0.5		3.5	76	4	79	58	0.7	
	0.3	0.6	76	4	63	64	0.8	
	0.3	0.6	60	4	54	56	0.9	
	0.6	1.4	76	4	77	106	1.4	
	0.6	1.4	76	4	51	104	1.4	
	1.0	2.2	76	4	57	101	1.3	
	1.5	3.4	76	3*	65	70	0.9	2
	1.5	3.4	76	4	70	45	0.6	

* Two females and one male.

the animals were able to make excellent growth without the added iron only in the absence of gossypol. However, there is reason to believe that during the absorption of material in the digestive tract, all of the iron would not be available for the precipitation of the gossypol and a considerable amount would be combined, possibly as an iron sulfide. Due to the insoluble nature of gossypol in weakly acid solutions, it does not seem likely that the gossypol would combine with the iron while in the stomach. If, on the other hand, the iron acted only as a catalytic agent for the oxidation of gossypol, one might expect 0.6 gm. of ferric citrate to be almost,

if not equally, as effective in bringing about the oxidation of the gossypol contained in 20 as in 10 gm. of seeds, and the quantitative relation between iron and gossypol which was accompanied with good growth would not be apparent.

The question therefore arose: How much iron will combine with gossypol under favorable conditions? It has been known for some time that these two substances react, but many difficulties have stood in the way of taking advantage of this reaction for the estimation of gossypol or even its identification. To answer this question, a quantity of gossypol was prepared from its acetate salt after the manner described by Carruth (8). After purification, this material was dissolved in alcohol in such amounts that 1 cc. of the resulting solution contained 1 mg. of gossypol. A number of iron standards were prepared, ferric ammonium sulfate and ferric citrate being used, and these used in a series of experiments to determine the weight of iron that would combine with 1 mg. of gossypol. In one series of experiments ferric ammonium sulfate was added in amounts which represented between 0.1 and 0.5 mg. of iron to 1 cc. portions of the gossypol solution. After the reaction had taken place, the excess iron was oxidized with $KMnO_4$ and determined colorimetrically in the diluted solution. The results of this work in which both gossypol and iron were varied within certain limits were not entirely satisfying, although indications were obtained which pointed to the formation of a definite compound of gossypol containing loosely bound iron.

In another set of experiments equal amounts of the ferric citrate solution were added to different amounts of gossypol contained in a dilute solution of alcohol made slightly alkaline with sodium hydroxide. Precipitation occurred almost immediately except when the concentration of either iron or gossypol was relatively low, and then only after standing a number of hours, or on neutralization of the excess alkali. The precipitates were filtered, washed, ignited, and analyzed for iron. Blanks were always run at the same time and a correction made for the amount of iron absorbed by the filter paper. Representative results obtained in these determinations have been presented below, since they lend support to the theory that iron combines with gossypol in amounts proportional to the quantity of gossypol present. However, when the amount of iron added was varied between wide limits, the

results were less consistent and until all the factors which control the above reaction have been carefully studied, no definite statement can be made as to the amount of iron that will combine with gossypol or completely precipitate it from its solution.

Gossypol.	Iron added.	Iron in precipitate.	Iron per mg. of gossypol.
mg.	*mg.*	*mg.*	*mg.*
1	0.5	0.052	0.052
2	0.5	0.111	0.055
3	0.5	0.175	0.058
4	0.5	0.222	0.055
5	0.5	0.269	0.054

As previously shown in the feeding experiments, between 0.14 and 0.22 gm. of iron was required to counteract the effects of 0.050 gm. of gossypol which was contained in diets composed of 10 per cent cotton seeds. The ratio of gossypol to iron might then be roughly expressed as 1:3, which, according to the above figures is all out of proportion with the experimental findings and a much wider range than should be necessary if all of the iron added were available for this reaction. In view of this, it seems reasonable to believe that an excess of iron must be present in the digestive tract to satisfy certain anions which either remove the iron from the gossypol-iron compound or precipitate the iron previous to its combination. There is also the possibility that the iron was performing some other and equally necessary function, although the amount of iron involved is far in excess of that required for normal metabolism. In fact, the animals were receiving daily over 10 times the amount of iron which Mitchell and Vaughn (9) supplied their rats to effect a recovery from nutritional anemia, and furthermore, no symptoms characteristic of iron deficiency have been observed in animals reared through several generations on cottonseed diets made up with autoclaved seeds. It should be pointed out that in no instance did the addition of iron, even in the most favorable amounts, to a diet containing 20 per cent raw seeds, account for as good growth of the animals as did those diets prepared with the equivalent amount of autoclaved seeds. Equally as good results have been obtained when the gossypol was removed by extraction as when destroyed by autoclaving.

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An attempt was made to determine the distribution of iron in the intestines of several of the animals with the hope of being able to identify the iron-gossypol compound. Such results as could be obtained, however, were subject to much criticism and were not of a nature which warranted reporting. The total amount one might expect to find in the entire intestinal contents would represent less than 10 mg. of gossypol, and until more delicate and reliable tests for this substance are discovered little emphasis could be placed on such findings.

One factor which seemed all important in attempting to express quantitatively the relation of iron to gossypol by means of the

TABLE III.

Food Consumed by Rats.

Nature of food.	Ferric citrate added.	Duration of period.	Average initial weight.	Average gain.	Average loss.	Average daily food intake.
	per cent	*days*	*gm.*	*gm.*	*gm.*	*gm.*
20 per cent cotton seeds.....		15	60		9	4.5
20 " " " "	1.5	15	122	21		12*
10 " " " "	0.6	12	140	17		11*
Basal diet.†.....	0.6	60	60	126		14
" "	1.5	40	62	85		12

* These measurements were made just previous to the close of the feeding experiments shown in Tables I and II.

† Basal diet composed of wheat 25, corn 40, milo 25, alfalfa 3, tankage 10, NaCl 1, and $CaCO_3$ 1.

growth records presented, was the amount of food consumed by the animals. Although a record was kept of the approximate amount of food consumed over each experimental period, the daily food intake was accurately measured only during certain intervals. It was to be expected that the iron salts which have a characteristic astringent taste, would in large amounts impair the palatability of the diets, and that the cotton seeds, which have a raw and somewhat unpleasant taste, would act similarly. In Table III the average daily food intake and the growth made by a number of the animals receiving diets of questionable palatability are presented.

As may be readily discerned, the animals receiving the raw seeds

in their diets without the addition of iron consumed but little and promptly declined. The decline and death of these animals cannot be taken as due to a refusal of the food because of its unpalatable nature, since the addition of the proper amount of iron, which is itself distasteful, brought opposite results. It seemed that the physical condition of these animals governed their food intake. The addition of 0.6 or 1.5 per cent ferric citrate to a basal diet did not prove injurious to the animals for several weeks, during which time the animals consumed the usual amount of food and showed no distaste for it.

TABLE IV.

Animals on Diets Containing Iron and 10 Per Cent Cotton Seeds.

Iron salt used.			Growth record.				
Ferrous ammonium sulfate.	Ferric ammonium sulfate.	Iron per 100 gm. of cotton seeds.	Duration of experiment.	No. of animals.	Average initial weight.	Average gain.	Average daily gain.
per cent	*per cent*	*gm.*	*days*		*gm.*	*gm.*	*gm.*
0.48		0.7	76	4	56	90	1.2
0.48		0.7	76	4	43	78	1.0
0.96		1.4	76	4	55	106	1.4
	0.59	0.7	76	4*	79	58	0.8
	0.59	0.7	60	4†	48	98	1.6

Average daily gain made by these two groups, 1.2 gm.

* Females.

† Males.

In order to meet certain objections which might be made to conclusions based on results obtained with only two iron salts, a similar study was made with the use of two other iron salts, one of which was a ferrous compound. The two salts used were ferrous and ferric ammonium sulfate, both of which are more readily soluble than the ferric citrate. If it is assumed that the value of iron in cottonseed diets is due in part to a precipitation of the gossypol, one might be led to believe that the reaction would be more complete in the presence of a very soluble iron salt such as one of the above, and as a consequence, less would be required. If such is true, it was not apparent in the results which are shown in Table IV.

The results show that approximately the same amount of iron

is required to offset the effects of the gossypol when added as the very soluble ferrous compound as when added as ferric citrate (see Table II), and when the amount of the former is decreased, growth is retarded. The ferric salt did not allow the animals to make normal growth when added in an amount which furnished less iron than the optimum amount found for ferric citrate.

It has often been observed in feeding cotton seeds to rats that after the animals begin to decline many of them will not respond to changes made in the diet. At other times when the injury has

TABLE V.

Growth of Rats on Diets Containing 20 Per Cent Cotton Seeds.

Rat No.	Initial weight.	5 days.		10 days.		15 days.		20 days.		Alterations in diet.
		Weight.	Daily food intake.	Weight.	Daily food intake.	Weight.	Daily food intake.	Weight.	Daily food intake.	
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
90	60	53	4	48	4	50*	4			1.5 per cent ferric citrate added 10th day.
91	60	58	4	50	4	52*	4			
92	70	60	4	55	6	60	7	65	7	1.5 per cent ferric citrate added 5th day.
93	75	65	4	85	7	85	7	95	8	
94	90	77	5	98	12	115	15	124	15	Changed to autoclaved seeds 5th day.
95	90	74	5	95	12	110	14	115	14	
96	90	75	4	95	12	110	14	118	14	Changed to autoclaved seeds and 1.5 per cent ferric citrate 5th day.
97	90	77	5	93	12	105	14	110	14	

* Died.

not progressed too far, as is often the case when the commercial meal instead of seeds is being used, the animals will promptly recover and resume normal growth. Similar observations have been made in large animal feeding. Since the iron salts proved so efficient in preventing the injury, it seemed desirable to determine if they would promote recovery in a similar manner. To do this and perhaps disclose the manner in which the iron acted as a protective agent against injury from gossypol, a number of feeding trials was carried out, cottonseed diets to which the iron salt was added after the animals had begun to show signs of gossypol

poisoning being used. In some instances the diets were changed at that time to ones in which autoclaved seeds were used. In Table V representative results of these feeding trials are presented with the growth made by the animals and the alterations made in their diets at 5 day intervals.

The results in Table V, as in Table III, point to the food intake of the animals being governed by the animals' response to the effects of the cotton seeds rather than to the proportion of cotton seeds in the diet. Rats 90 and 91 clearly show the loss of appetite and rapid decline in weight characteristic of animals undergoing severe gossypol poisoning. The addition of iron to their diets on the 10th day did not bring about recovery and the animals died 4 days later. No doubt the effects of the gossypol had progressed so far during this time that the addition of iron was not of itself sufficient to promote recovery unless some other alterations were made in the diet. When this preliminary feeding period was shortened to 5 days and the iron then added, the animals (Rats 92 and 93) showed some signs of recovery, although their food intake remained low and they never displayed the healthy condition of normal rats. However, when the diet was changed at the end of a 5 day preliminary feeding period (Rats 94 and 95), recovery was quite rapid and the animals were able to resume growth without further additions of iron to the diet. These results, when considered in their relation to those in Tables I and II, point to the value of iron as a means of preventing or delaying the injury from gossypol, but not providing a very effective cure. As to the manner in which the iron acts, there is no direct proof, although at the present time it seems reasonable to believe that the iron combines with the gossypol to form an insoluble compound which is slowly, or not at all, absorbed in the digestive tract. Since rats do not exhibit all the manifestations of gossypol poisoning of larger animals, namely diarrhea, paralysis, respiratory disturbances, and often blindness, it might be well to repeat certain parts of this work with other animals in which loss of appetite and inanition are not so pronounced. It is believed that the postmortem examinations, which will be made in connection with the feeding work mentioned in the earlier part of this paper, will yield important results which bear directly on the use of iron in cottonseed meal feeding.

SUMMARY AND CONCLUSION.

A study has been made of the value of iron salts in preventing the poisoning of animals by gossypol, the toxic principle in cotton seeds, and the extent to which such salts may serve to promote the recovery of animals so poisoned. The iron salts used were ferric oxide, ferric citrate, ferric ammonium sulfate, and ferrous ammonium sulfate. Gossypol injury was produced by feeding to the test animals, which were albino rats, diets containing either 10 or 20 per cent cotton seeds of known gossypol content. Growth and general appearance of the animals as used in previous studies with gossypol were employed to determine the extent of the injury and the efficiency of the different iron salts in counteracting it.

The animals receiving the smaller amount of cotton seeds in their diet made poor growth and toward the end of the experiment displayed a decidedly unhealthy condition; those receiving the larger amount promptly declined in weight and several died. The addition of ferric oxide to the diets was of no value (probably due to its insolubility) in counteracting the effects of the gossypol; but ferric citrate and ferrous ammonium sulfate, when present in sufficient amounts to furnish 3 gm. of iron per 1 gm. of gossypol, were equally effective in allowing the animals to make nearly normal growth for some time. The addition of smaller amounts of these two salts, or of ferric ammonium sulfate, was attended with less favorable growth.

Food intake records showed that the retarded growth of the animals on the raw cottonseed diets was due not only to the effects of gossypol present, but also to a refusal by the animals to consume much of the feed. Food consumption was increased by the addition of ferric citrate.

The results with ferric citrate pointed to a quantitative relationship existing between the amount of gossypol in the diet and the amount of iron necessary to counteract its toxic effects. Experiments in the test-tube also showed that the reaction between gossypol and iron is of a quantitative nature. It is concluded from these studies that iron delays the effects of cottonseed injury by combining with the gossypol to form an insoluble compound. A change in diet is more effective than the addition of iron for promoting recovery from gossypol poisoning.

1. Wi
2. Wi
3. Wi
4. Mc
5. Gal
6. Sch
7. Gal
8. Car
9. Mit

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BIBLIOGRAPHY.

1. Withers, W. A., and Brewster, J. F., *J. Biol. Chem.*, 1913, xv, 161.
2. Withers, W. A., and Carruth, F. E., *J. Agric. Research*, 1915, v, 216.
3. Withers, W. A., and Carruth, F. E., *J. Biol. Chem.*, 1917, xxxii, 245.
4. McGowan, J. P., and Crichton, A., *Biochem. J.*, 1924, xviii, 265.
5. Gallup, W. D., *J. Biol. Chem.*, 1928, lxxvi, 43.
6. Schwartze, E. W., and Alsberg, C. L., *J. Agric. Research*, 1924, xxviii, 173.
7. Gallup, W. D., *Ind. and Eng. Chem.*, 1927, xix, 726.
8. Carruth, F. E., *J. Am. Chem. Soc.*, 1918, xl, 647.
9. Mitchell, H. S., and Vaughn, M., *J. Biol. Chem.*, 1927, lxxv, 123.

THE RESPIRATORY PROTEINS OF THE BLOOD.

III. THE ACID-COMBINING CAPACITY AND THE DIBASIC AMINO ACID CONTENT OF THE HEMOCYANIN OF LIMULUS POLYPHEMUS.

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(Received for publication, March 16, 1928.)

The maximal acid-combining capacity of the hemocyanin of the horseshoe crab, *Limulus polyphemus*, has been measured and found to agree closely with the value predicted from the dibasic amino acid content of this protein.

In accordance with the polypeptide linkage of amino acids in the protein molecule, the dibasic amino acids should each retain one free basic group available for combination with acid, and the dicarboxylic amino acids should similarly each retain one free carboxyl group capable of uniting with base. A quantitative relationship can, therefore, be predicted between the composition of proteins and their maximal acid- and base-binding power. In collecting the data bearing on this relationship, Cohn (1925) has been able to demonstrate clearly that those proteins that are rich in basic amino acids have high acid-combining capacities, while those that are poor in this respect have low acid-combining capacities. The same relationship appears to hold true for the dicarboxylic acids and the base-binding power. In many instances the agreement between the acid- or base-binding power as measured and as predicted from the amino acid components is extraordinarily close. Chapman, Greenberg, and Schmidt (1927) also have found that the capacity of several proteins to combine with acid dyes can be correlated with their content of arginine, lysine, and histidine. Recently Lewis (1927) has demonstrated the correspondence between the diamino acid content of hemoglobin and its maximal acid-binding power. He found that

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1 equivalent (16,660 gm.) of hemoglobin bound 24 or 25 mols of acid, which would correspond to 144 or 150 $\times 10^{-5}$ mols per gm. Cohn (1925) had previously estimated an acid-binding power of 146 $\times 10^{-5}$ mols per gm. from the histidine, arginine, and lysine of hemoglobin as determined by Van Slyke (1911).

Measurement of Maximal Acid-Binding Power of Hemocyanin.

The hemocyanin used in the present experiments was prepared by Mrs. Mary A. Shotts and Miss Elizabeth Ingalls. Two distinct methods were employed in purifying material that had been prepared some months previously by precipitation from the serum with ammonium sulfate at half saturation. Specimen XII A was repeatedly salted out with ammonium sulfate, and redissolved, and then dialyzed against dilute sodium hydroxide, as described by Redfield, Coolidge, and Shotts (1928). It was not washed at the isoelectric point. Specimen XV A was prepared similarly except that ammonium hydroxide was used to render the dialyzing solution alkaline. The other three preparations were freed of most of the ammonium sulfate by dialysis, then precipitated at the isoelectric point by the addition of 0.01 N hydrochloric acid, and washed by repeated decantation with a large volume of water. The precipitate was then redissolved by adding a small quantity of 0.1 N sodium hydroxide.

The purity of the hemocyanin is indicated by the copper contents of the specimens. Specimens XII A, XIV A, and XV A contained respectively 0.167, 0.169, and 0.168 per cent of the dry weight as copper. The most pure preparations of Redfield, Coolidge, and Shotts yielded 0.173 per cent copper.

The samples used in the determinations were made up by diluting small measured quantities of these preparations with distilled water containing known quantities of hydrochloric acid. The concentration of hemocyanin in each specimen is shown in Column 2 of Table I. The total concentration of hydrochloric acid is entered in Column 3. This may be divided into three portions: (*a*) that which is required to neutralize the base present in the preparation, (*b*) that which is uncombined and exists as free hydrochloric acid, (*c*) that which is bound by combination with the hemocyanin. The latter quantity may be obtained by sub-

tracting from the total hydrochloric acid the sum of the two former, both of which are measurable.

The base present in the material as the result of the method of preparation was measured by determining how much 0.01 N hydrochloric acid must be added to produce maximal precipitation

TABLE I.

Preparation No. (1)	Concentration of hemocyanin. (2)	Concentration of HCl. (3)	Concentration of combined base. (4)	pH (5)	Log $\frac{1}{\gamma}$ (6)	Free HCl. (7)	Bound HCl. (8)	HCl bound per gm. of hemocyanin. (9)
	gm. per l.	*mols per l.*	*mols per l.*			*mols per l.*	*mols per l.*	*mols*
XII A	10.05	0.0152	0.0026	3.072 (2)*	0.043	0.0009	0.0117	116×10^{-5}
	10.05	0.0202	0.0026	2.598 (2)	0.047	0.0028	0.0148	147
	10.05	0.0252	0.0026	2.234 (8)	0.048	0.0065	0.0161	160
	10.05	0.0303	0.0026	2.003 (10)	0.050	0.0112	0.0165	164
	10.05	0.0379	0.0026	1.768 (2)	0.053	0.0193	0.0160	159
	10.05	0.0405	0.0026	1.718 (6)	0.053	0.0216	0.0163	162
	10.05	0.0455	0.0026	1.628 (1)	0.055	0.0267	0.0162	161
XIV A	11.50	0.0300	0.0009	1.985 (11)	0.050	0.0116	0.0175	152
	11.50	0.0400	0.0009	1.727 (4)	0.053	0.0212	0.0179	156
XIV B	12.00	0.0400	0.0014	1.745 (9)	0.053	0.0200	0.0186	155
XIV C	14.30	0.0360	0.0012	1.969 (3)	0.053	0.0121	0.0227	159
	14.30	0.0400	0.0012	1.864 (5)	0.053	0.0155	0.0233	163
XV A	16.00	0.0400	0.0053	2.122 (3)	0.053	0.0085	0.0262	164
	16.00	0.0480	0.0053	1.856 (4)	0.055	0.0158	0.0269	168
	16.00	0.0560	0.0053	1.677 (4)	0.058	0.0240	0.0267	167
	16.00	0.0720	0.0053	1.442 (3)	0.065	0.0420	0.0247	154

* The number in parentheses is the number of determinations averaged to secure the given value of pH.

at the isoelectric point. The precipitation zone is sufficiently sharp to enable this to be done with an accuracy of 2×10^{-5} mols per gm. of hemocyanin. The base combined with the preparations is entered in Column 4 of Table I.

The concentration of free hydrochloric acid (HCl) was esti-

mated from the hydrogen potential, pH, of the solution. Since $pH = \log \frac{1}{\gamma(H^+)}$ and $(HCl) = (H^+)$ on the theory of complete electrolytic dissociation,

$$\log \frac{1}{(HCl)} = pH - \log \frac{1}{\gamma} \quad (1)$$

The hydrogen potential was measured by means of the hydrogen electrode. The sample was introduced into a Clark electrode vessel and rocked for ½ hour at a temperature of approximately 25° before the potential was measured. The pH values so obtained are entered in Column 5 of Table I. The values of $\log \frac{1}{\gamma}$, corresponding to the total concentration of HCl in the sample, are taken from data given by Cohn and Berggren (1924) and are entered in Column 6. Since the values of γ are estimated on the assumption that the electromotive force of the 0.1 N calomel electrode is 0.3356 volt at 25° following Lewis, Brighton, and Sebastian, this value has been employed in estimating the hydrogen ion activities of the hemocyanin solutions. The concentration of free hydrochloric acid, as calculated from equation (1), occupies Column 7. The acid bound by the hemocyanin, entered in Column 8 of Table I, is obtained by subtracting from the total concentration (Column 3) the concentration of acid required to bring the preparation to its isoelectric point (Column 4) plus the concentration of free hydrochloric acid (Column 7). By dividing this result by the gm. of hemocyanin in a liter of solution (Column 2) the acid bound is expressed in terms of mols per gm. of hemocyanin.

These figures indicate that at pH values of 2.23 and less the acid bound by *Limulus* hemocyanin does not increase to a detectable degree even though the free acid is increased to nearly double the quantity bound. The most representative value for the series of measurements is 160×10^{-5} mols per gm.

It may be pointed out here that on the addition of hydrochloric acid to *Limulus* hemocyanin the solution becomes colorless and loses its capacity to combine with oxygen.

An attempt to measure the maximal base-binding power of

Limulus hemocyanin has been unsuccessful. The protein appears to undergo hydrolysis on the addition of sodium hydroxide before the combined base becomes constant.

Titration of Hemocyanin with Hydrochloric Acid.

When quantities of hydrochloric acid, smaller than that sufficing to combine with all the available basic groups, are added to

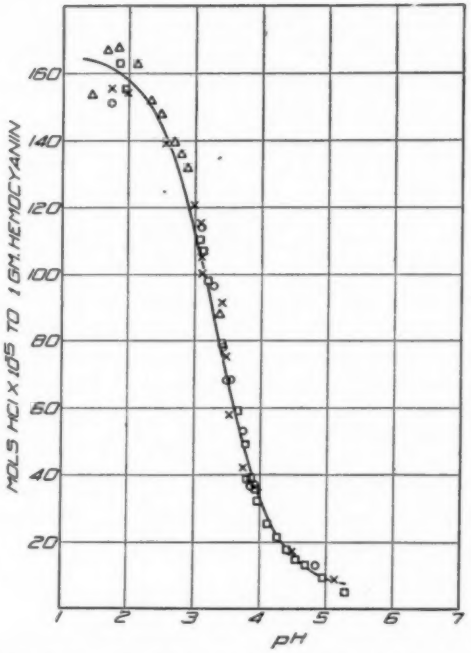

FIG. 1. Titration curve of *Limulus* hemocyanin with hydrochloric acid at 22–25°. The ordinates measure the quantity of hydrochloric acid combined with the protein. The different symbols refer each to a different preparation of the protein.

Limulus hemocyanin, the hydrogen potential of the solution varies with the amount of acid combined with the protein in a way illustrated in Fig. 1. The titration curve is a symmetrical sig-

moid very similar throughout its length to that of serum albumin (Cohn, 1925). The data are quantitatively described by a curve drawn on the assumption that hemocyanin combines with acid as a univalent base, that is, that all the basic groups behave alike and independently of each other. The curve drawn through the points in Fig. 1 is constructed from the Henderson-Hasselbalch equation. In order to obtain the best fit between the theoretical curve and the data it has been assumed that basic groups equivalent to 6×10^{-5} mols per gm. of hemocyanin combine in reactions taking place near the isoelectric point and consequently do not behave like the remainder. A maximal acid-binding capacity of 166×10^{-5} mols per gm. has been employed because most of the

TABLE II.

(1)	Percentage of total N attributable to basic amino acid. (2)	Basic amino acids in hemocyanin, mols $\times 10^{-5}$ per gm. (3)	Equivalence of basic amino acids to copper atoms. (4)	Probable No. of basic amino acids in hemocyanin molecule. (5)
	per cent			
Arginine	15.73	48.6	17.8	36
Histidine	13.23	54.5	20.0	40
Lysine	8.49	52.4	19.2	38
Total basic amino acids		155.5	57.0	114
Total acid-combining capacity		160	58.7	117

higher points on the curve were obtained with preparation XV A which yielded a value of this magnitude. The value of the apparent dissociation constant, pK, is 3.3.

Dibasic Amino Acids of Hemocyanin.

Van Slyke (1911) has determined the percentage of nitrogen in *Limulus* hemocyanin attributable to various amino acids. In Table II we have entered his figures (the average of two separate analyses) for arginine, histidine, and lysine. From these figures we have calculated the mols of each dibasic amino acid present per gm. of hemocyanin.[1] The sum of these is 155.5×10^{-5} mols

[1] These values differ slightly from those estimated by Cohn (1925) from Van Slyke's data. This is because he employed the value 16.18 as the per

per gm. of hemocyanin. The agreement of this figure with the direct measurement of the total acid-binding power is satisfactory when the accuracy of the methods involved are taken into account.

Redfield, Coolidge, and Shotts (1928) have shown that 36,700 gm. of hemocyanin contain 1 gm. atom of copper. Multiplying this figure by the dibasic amino acid content of 1 gm. of hemocyanin one obtains the equivalence of these components to the copper as shown in Column 3 of Table II. Inasmuch as there are probably 2 atoms of copper in the molecule of *Limulus* hemocyanin, the probable number of dibasic amino acids of each sort present may be estimated as the nearest integer to twice the equivalent value, as shown in Column 4. Similarly the equivalent acid-combining capacity of the entire molecule may be estimated as 117 from the measurements reported in this paper.

SUMMARY.

1. The hemocyanin of *Limulus polyphemus* binds about 160×10^{-5} mols of acid per gm.

2. It reacts with acid as though it were a univalent base with an apparent dissociation constant of 3.3.

3. The maximal acid-binding power agrees closely with that calculated from the dibasic amino acids of this protein.

4. The probable number of molecules of arginine, histidine, and lysine in the hemocyanin molecule, and its total number of acid-binding groups are estimated.

BIBLIOGRAPHY.

Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A., *J. Biol. Chem.*, 1927, lxxii, 707.

Cohn, E. J., *Physiol. Rev.*, 1925, v, 349.

Cohn, E. J., and Berggren, R. E. L., *J. Gen. Physiol.*, 1924, vii, 45.

Lewis, P. S., *Biochem. J.*, 1927, xxi, 46.

Redfield, A. C., Coolidge, T., and Shotts, M. A., *J. Biol. Chem.*, 1928, lxxvi, 185.

Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.

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cent of nitrogen in *Limulus* hemocyanin as obtained by Alsberg and Clark. Redfield, Coolidge, and Shotts (1928) found carefully purified hemocyanin to contain 17.3 per cent of nitrogen. This value has consequently been employed in calculating Table II.

EFFECT OF INTRAPERITONEAL INJECTIONS OF INSULIN UPON THE BLOOD SUGAR OF WELL FED RABBITS.

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(Received for publication, February 29, 1928.)

Hoskins and Snyder (1) reported that in well fed rabbits, insulin injected intraperitoneally is in most cases not followed by a lowering of the blood sugar such as occurs in fasting animals similarly treated. Their conclusion was based upon experiments performed on a series of rabbits, well fed and chosen at random. They used twenty-eight different rabbits in a total of thirty-one experiments. In their table, representing the results obtained from the experiments performed upon the well fed animals, thirteen showed a marked lowering of the blood sugar. Of the twenty-eight rabbits used, the experiments were repeated with three only, two of which showed a marked lowering of the blood sugar the second time.

In a paper from this laboratory (2), we called attention to the great resistance to insulin shown by "uneducated" rabbits. We observed that in general it requires from 3 to 5 units of insulin per kilo of body weight, used intravenously, to produce hypoglycemic convulsions in uneducated rabbits. In some instances, an intravenous dose of from 8 to 10 units per kilo was required. However, by educating these rabbits, *i.e.* by keeping them on a strict regimen and by giving them convulsive doses of insulin every 7th day after a 24 hour fast, the amount of insulin required was greatly diminished. In the course of a few months, such rabbits become stabilized and respond to a smaller and more definite dose. In this connection we emphasized the importance of administering a convulsive dose every 7th day, for when this rule is not followed, there is a tendency to develop immunity. In a few of our cases

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we were able to develop immunity to such a marked degree that a total intravenous dose of 50 units did not produce convulsions.

The results of the intraperitoneal injections of insulin reported by Hoskins and Snyder were so striking that we were led to repeat this work paying special attention to diet and to other factors known to influence the response to insulin.

General Procedure.

The rabbits used consisted of four young uneducated males, four young uneducated females, two females previously used for insulin work, and three standard insulin females.

In another paper from this laboratory (3), it was shown that animals fed a high carbohydrate, acid-forming diet, require more insulin to produce hypoglycemic convulsions than those eating a low carbohydrate, basic diet. Our uneducated rabbits were, therefore, divided into two groups. The young males, Rabbits 1 to 4, were given a high carbohydrate diet. The females, Rabbits 5 to 8, were fed our regular, low carbohydrate diet. In all experiments, the well fed rabbits were given an abundant food supply until the initial blood samples were taken. They were then given insulin and left in wire cages without food. The insulin was injected into the peritoneal cavity about half-way between the umbilicus and symphysis pubis. Blood samples were obtained from the marginal ear vein and blood sugar was estimated by the method of Folin and Wu.

It was found necessary to use different doses of insulin than were administered by Hoskins and Snyder. Lilly's insulin, U-100, was used. This was diluted 1:100, 1:50, and 1:25 before injection. The same sample of insulin was used for all the experiments. A dose of 3 units per kilo did not appear sufficient for young, well fed rabbits, especially for those not educated to insulin and being maintained upon a high carbohydrate diet. Larger amounts of insulin were consequently given.

EXPERIMENTAL.

Series 1.—In Table I are reported the findings obtained with the four young uneducated male rabbits fed the high carbohydrate diet. The average weight of these animals was about 2 kilos when the experiments began. For the first injection Rabbits 1

TABLE I.

Changes in Blood Sugar of Young "Uneducated" Male Rabbits Fed High Carbohydrate Diet.

Date.	Rabbit No.	Weight.	Insulin.	Form of administration.	Blood sugar, mg. per 100 cc.				
					Initial.	1 hr.	2 hrs.	4 hrs.	6 hrs.
1927		*kg.*	*units per kg.*						
Dec. 14	1	2.25	3	Intravenous.	105	79	75		
	2	1.95	3	Subcutaneous.	83	66	50		
	3	2.20	3	Intraperitoneal.	100	118	130	100	
	4	2.05	3	"	108	118	130	130	
Dec. 21	1	2.40	3	"	100		34	50	78
	2	2.00	3	"	105		55	71	100
	3	2.35	3	"	103		50	66	95
	4	2.15	3	"	103		42	38	91
Dec. 29	1	2.50	3	"	100		55	67	
	2	2.15	3	"	95		67	80	
	3	2.50	3	"	100		52	67	
	4	2.30	3	"	95		56	80	
1928									
Jan. 4	1	2.55	5*	Intravenous.					
	2	2.00	5*	"					
	3	2.50	5*	"					
	4	2.20	5*	"					
Jan. 12	1	2.60	8	Intraperitoneal.	111		58	49	
	2	2.10	8	"	95		55	55	
	3	2.50	8	"	118		57	80	
	4	2.30	8	"	91		52	65	
Jan. 19	1	2.65	8	Subcutaneous.	114		72	60	
	2	2.25	8	"	121		57	72	
	3	2.50	8	"	114		68	50	
	4	2.30	8	"	125		68	70	
Jan. 26	1	2.75	8	Intravenous.	111	67			
	2	2.30	8	"	108	70			
	3	2.60	8	"	125	87			
	4	2.35	8	"	108	65			

* No blood sugar tests were performed. None of this group showed any symptoms of hypoglycemia.

and 2 were given an intravenous and a subcutaneous dose, respectively. They showed low blood sugar 1 hour after the injection and maintained this level for over 2 hours. Rabbits 3

and 4 which received intraperitoneal injections showed no lowering of the blood sugar. 1 week later the intraperitoneal injections were performed upon all four of the rabbits. The insulin caused a hypoglycemia which continued for over 4 hours. The following week the experiments were repeated in a similar manner and with similar results. On January 4, an intravenous dose of 5 units per kilo was tried in an effort to obtain insulin convulsions. Blood

TABLE II.

Changes in Blood Sugar of Young "Uneducated" Female Rabbits Fed Low Carbohydrate Diet.

Date.	Rabbit No.	Weight.	Insulin.	Form of administration.	Blood sugar, mg. per 100 cc.					Time to convulsion.
					Initial.	1 hr.	110 min.	2 hrs.	4 hrs.	
1918		*kg.*	*units per kg.*							*min.*
Jan. 4	5	2.00	5	Intraperitoneal.	100			37	80	180
	6	2.10	5	"	103			33		132
	7	2.95	5	"	105			118	111	
	8	2.75	5	"	100			29		113
Jan. 11	5	1.80	4	"	100					106
	6	2.00	4	"	114			50		150
	7	3.00	6	"	118			40	47	
	8	2.75	4	"	125			50		200
Jan. 18	5	2.00	4	Intravenous.	100	68				
	6	2.25	4	"	95	62				
	7	3.15	6	"	118	59				
	8	3.00	4	"	118	60				
Jan. 25	5	2.00	4	Subcutaneous.	90					90
	6	2.30	4	"	105		28			110
	7	3.20	6	"	100		30			115
	8	3.00	4	"	103		40			120

sugar tests were not performed as no symptoms of weakness developed. 1 week later a dose of 8 units per kilo was given intraperitoneally. A lowering of the blood sugar took place, unaccompanied by an insulin reaction which might have been expected from the injection of so large a dose. Upon trying this dose of 8 units per kilo subcutaneously, on January 19, similar results were obtained. The intravenous injection of an equal number of units, however, did not produce so marked a lowering

of the blood sugar as was obtained from the intraperitoneal and the subcutaneous injections.

Series 2.—In our second series of experiments, the four young uneducated female rabbits were used. They were kept on our regular standard low carbohydrate diet, consisting of alfalfa hay, with the addition of some barley on the day they received their insulin and on the one following. They were well fed until blood samples were taken, and were then injected intraperitoneally

TABLE III.

Changes in Blood Sugar of Mature Female Rabbits Fed High Carbohydrate Diet.

Date.	Rabbit No.	Weight.	Insulin.	Form of administration.	Blood sugar, mg. per 100 cc.						
					Initial.	1 hr.	1.5 hrs.	2 hrs.	4 hrs.	5 hrs.	6 hrs.
1927		*kg.*	*units per kg.*								
Dec. 15	54	3.75	3	Intraperitoneal.	100	61		55	37	40	105
	57	3.60	3	"	103	66		52	83	111	
Dec. 22	54	4.00	1.5	"	114			47	79		105
	57	3.65	1.5	"	125			71	50		100
Dec. 29	54	4.00	1.5	"	108			42	80		
	57	3.70	1.5	"	100			37	56		
1928											
Jan. 12	54	4.00	4	"	111			30	40*		
	57	3.70	4	"	110			87	100		
Jan. 20	54	4.25	4	Subcutaneous.	108		52		91		
	57	3.85	8	Intraperitoneal.	105		54		75		

* This rabbit had convulsions in 150 minutes. It was revived with 5 cc. of 20 per cent glucose; nevertheless it showed low blood sugar at 4 hours.

with the diluted insulin. The procedure already described was followed and our findings are reported in Table II. Each animal was injected with 5 units per kilo. This dose proved to be almost fatal to three of the four rabbits. They were violently convulsed. A subcutaneous injection of 5 cc. of 20 per cent glucose had little effect as to their immediate recovery. They were given another 5 cc. of the same glucose solution and even then did not recover for 30 minutes. Only Rabbit 7 did not manifest any lowering of the blood sugar after this first injection. The week following,

each animal received 4 units per kilo intraperitoneally instead of 5, with the exception of Rabbit 7 which received 6 units. The three which were convulsed previously were again convulsed. Rabbit 7, moreover, showed symptoms of hypoglycemia and a marked lowering of the blood sugar. Upon repeating the last dose intravenously, none of the rabbits was convulsed. They

TABLE IV.

Changes in Blood Sugar of Standard Insulin Rabbits.

Date.	Rabbit No.	Weight.	Diet.	Insulin.	Form of administration.	Intravenous factor.	Blood sugar, mg. per 100 cc.				Time to convulsion.
							Initial.	1 hr.	2 hrs.	4 hrs.	
1927		*kg.*		*units per kg.*							*min.*
Dec. 16	19	3.90	Low carbohydrate.	3	Intraperitoneal.	0.6	118	30			75
	112	4.50	" "	3	"	1.2	118	29			88
	114	3.65	" "	3	"	1.4	121	32			95
Dec. 23	19	3.71	" "	1.5	"	0.6	100	40	33	57	
	112	4.70	" "	1.5	"	1.2	121	36			100
	114	3.75	" "	1.5	"	1.4	114	31			105
Dec. 30	19	3.50	24 hr. fast.	0.4	"	0.6	103		30		150
	112	4.55	24 " "	0.5	"	1.2	108		22		127
	114	3.75	24 " "	0.5	"	1.4	100		20		140
1928											
Jan. 3	120	4.00	24 " "	0.35	"	0.9	98		43	87	
	121	4.00	24 " "	0.3	"	0.6	95		43	79	
	12	4.20	24 " "	0.3	"	0.6	108		37		120
	78	3.30	24 " "	0.45	"	0.7	100		33		115
Jan. 6	112	4.90	High carbohydrate.	0.5	"	1.2	151		70	98	
	114	3.90	" "	0.5	"	1.4	154		80	100	

showed, however, a lowering of the blood sugar. The subcutaneous injections resulted in hypoglycemic convulsions in all cases.

Series 3.—For this experiment two female rabbits were used, upon which insulin experiments had previously been performed. Their average weight was over 3.6 kilos. Their diet until December 15 consisted chiefly of alfalfa hay, with barley once a week.

After that date they were put on the high carbohydrate diet. In Table III we report our findings. These animals were well fed before each experiment. Upon receiving 3 units per kilo intraperitoneally there was a lowering of the blood sugar for the duration of 5 hours in Rabbit 54 and 4 hours in Rabbit 57. On December 22 and 29 they were given, in a similar manner, 1.5 units per kilo. On January 12, they were injected with 4 units per kilo. Rabbit 54 had an insulin convulsion in 2.5 hours. A subcutaneous injection of 4 units per kilo produced only a lowering of the blood sugar in this rabbit.

Series 4.—This experiment was performed upon three of our standard rabbits. These animals had been educated to insulin and responded to a certain definite dose. The figures in Table IV under "intravenous factor" represent the percentage of 1 unit of insulin per kilo required to produce convulsions. There was only one deviation from their low carbohydrate diet, which occurred on January 6, when Rabbits 112 and 114 received by mistake an extra portion of barley. Rabbit 19 died from pneumonia during the 1st week of January. After the injection of 3 and 1.5 units per kilo intraperitoneally, severe convulsions took place. Following a 24 hour fast 0.5 unit per kilo was sufficient to produce convulsions, while more than 1.2 units per kilo injected intravenously would have been required. See Table IV for results.

DISCUSSION.

The experimental conditions of Series 1 were comparable with those of Hoskins and Snyder. Like them, we found no hypoglycemia in two rabbits after the intraperitoneal injection of 3 units per kilo. The 2 succeeding weeks, however, all four of these animals showed marked hypoglycemia with the same dose. Subsequent experiments revealed that these rabbits were able to withstand 8 units per kilo without convulsions. It made no difference whether the insulin was given intraperitoneally, subcutaneously, or intravenously.

The results of the experiments of Series 2 show anew the greater susceptibility of rabbits living upon a low carbohydrate, basic diet. 5 units of insulin given intraperitoneally produced severe convulsions in three of the animals, while one had no hypoglycemia. The following week, 4 units per kilo caused convulsions

in the same animals and the other responded with hypoglycemia. Corresponding amounts of insulin administered subcutaneously produced convulsions in all of the animals. When the same doses were given intravenously, there was hypoglycemia at 1 hour, but no convulsions resulted. The first two series of experiments show that with well fed uneducated rabbits, there may be no hypoglycemia following the first intraperitoneal injection of insulin. Subsequent doses are effective even though the animals are well fed.

The results of Series 3 and 4, in which rabbits accustomed to insulin were used, show that they responded to the first intraperitoneal injection. Smaller doses were also required. Furthermore, the intraperitoneal injections were more effective than the intravenous in the cases of our standard, fasted animals.

It is evident that our results do not accord with the findings of Hoskins and Snyder. Like them, however, we found that sometimes there was no hypoglycemia after the injection of insulin into well fed rabbits. Subsequent injections were, nevertheless, effective. In fact, our results indicate that the intraperitoneal route is more efficient than the intravenous and about on a par with the subcutaneous. It seems unnecessary, therefore, to theorize about the presence of some substance in the liver which buffers the action of insulin administered intraperitoneally. The fact that sometimes there is no hypoglycemia following the first intraperitoneal injection in well fed rabbits is puzzling. We have no explanation to offer.

SUMMARY.

The first intraperitoneal injection of insulin into well fed rabbits sometimes fails to produce hypoglycemia. Subsequent injections, however, produce results very comparable to those following subcutaneous injections. Intraperitoneal injections are more effective than are the intravenous.

BIBLIOGRAPHY.

1. Hoskins, F. M., and Snyder, F. F., *J. Biol. Chem.*, 1927, lxxv, 147.
2. Sahyun, M., and Blatherwick, N. R., *Am. J. Physiol.*, 1926, lxxvi, 677.
3. Blatherwick, N. R., Long, M. L., Bell, M., Maxwell, L. C., and Hill, E., *Am. J. Physiol.*, 1924, lxix, 155.

CONCERNING THE SIMILARITY OF GLUKHORMENT AND SYNTHALIN.

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Von Noorden (1) has recently reported his clinical experience with a new hypoglycemic principle, marketed as "glukhorment." Meissner is credited with the development of this product, which, according to his patent, is produced by fermenting endocrine glands until glycocyamine results. In a second patent application glycocyamine or a similar working compound is added. Von Noorden considers the latter procedure of theoretical, patentable significance and mentions that in the preparation which he used for his clinical trials, and which is on the market, no addition of glycocyamine, guanidine, or even synthalin was made. He doubts whether the traces of guanidine compound in the preparation function as the blood sugar-lowering principle. However, if the active principle should prove to be a guanidine derivative, he feels it must have a much more powerful effect than synthalin.

In this paper, evidence is presented which indicates that the guanidine derivative existing in the glukhorment is either synthalin or a near homologue of it. The chloroplatinate, chloroaurate, and picrate of the guanidine base isolated from glukhorment melt within 2–1° of the same salts made from synthalin. The analytical values of the platinum salt for nitrogen and platinum are also in very close agreement, when one considers that the glukhorment base must be separated from contaminating protein, fat, and phospholipid materials. Tested upon rabbits the glukhorment base had exactly the same effect as synthalin. 8 mg. of base per kilo given intravenously produced a hypoglycemia in 2 hours and convulsions the 4th hour. Glucose failed to restore the animal

and death ensued. 4 mg. of base per kilo had a slower action, the animal dying within 12 hours. 3 and 2 mg. per kilo produced no hypoglycemia and no general effect. The same amount of synthalin, 4 or 5 mg. per kilo parenterally injected, is the minimum effective dose in rabbits (2, 3). The glukhorment base also interferes with deaminization and injures the kidneys as does synthalin (Blatherwick, Sahyun, and Hill (2)). These effects are shown in Table I.

Both synthalin and the glukhorment base give a very intense red coloration with the diacetyl or the α-naphthol-hypochlorite

TABLE I.

Blood Changes Following Injection of Glukhorment Extracts and Glycine.

Date.	Time.	Blood content.			Remarks.
		Sugar.	Urea N.	Amino acid N.	
1927		*mg.*	*mg.*	*mg.*	
Rabbit 407.					
Sept. 16	9.50 a.m.	121	29.4	8.0	At 9.50 a.m. 6 cc. crude extract of glukhorment and 1 gm. glycine per kilo subcutaneously.
	11.20 "	138	34.5	53.5	
	12.40 p.m.	105	33.3	48.5	
	2.30 "	37	43.0	52.5	
Rabbit 303.					
Sept. 17	9.20 a.m.	121	24.0	8.9	At 9.20 a.m. 27 mg. crude extract of glukhorment and 1 gm. glycine per kilo subcutaneously. Died during night.
	10.50 "	125	23.5	33.3	
	12.10 p.m.	114	28.5	35.0	
	3.00 "	67	34.5	37.0	
	4.35 "	67	36.0	43.0	

test, quite different from the pink obtained with glycocyamine. The content of the guanidine base in the tablets is not so low as von Noorden suspected. 1 gm. of the purified base was isolated from 400 tablets. Since the separation is by no means quantitative, four tablets of glukhorment, the daily dose recommended for clinical use, may well-nigh approach the clinical dose for synthalin.

Fränkel and Friedmann (4) have recently isolated a dodecane diaminodicarboxylic acid from the tryptic digestion of casein. It is possible to conceive of this compound being decarboxylated

by bacteria to diamino decane, the parent substance of synthalin. This suggestion is offered as a possible explanation for the occurrence of synthalin in the glukhorment tablets.

Isolation and Purification of Guanidine Derivative.

The glukhorment tablets contain a large amount of protein material. Some of this is soluble in water, yielding a solution with a pH of 6.4, from which phosphotungstic acid throws down a precipitate. Still more is soluble in acidulated water. These solutions give positive biuret and negative purine base tests. In order to avoid contaminating the guanidine derivative with protein, an absolute alcohol extraction was used to effect a separation. A trial extraction showed that much fatty material was leached out by the alcohol. In order to avoid contamination with fat, the alcohol extraction was preceded by an ether extraction. In following the course of the guanidine derivative in the separation, the qualitative test developed by Sakaguchi (5) was used. It is difficult to obtain a good positive test for guanidine on aqueous extracts of the glukhorment tablets. The alcoholic extract gives an intense red color, however, and from this step on to the isolation of the guanidine base, the test served whenever it was necessary to locate the compound in a precipitate or a filtrate. The alcoholic extract contains in addition to the guanidine compound some phospholipid material, which was insoluble in boiling water and could therefore be separated. The aqueous solution of the guanidine compound substantially freed from protein, fat, and phospholipid materials, was dark in color and gave a positive test for phosphates. An alkaline silver separation, the conventional method of isolating guanidine compounds from biological materials, was used for further purification.

Details of Isolation.—440 glukhorment tablets were extracted for a day in a Soxhlet with 500 cc. of absolute ether. The extracted fatty material was discarded. The tablets were then extracted with 500 cc. of absolute alcohol for 3 days. The alcohol was evaporated to dryness and the residue was taken up with 500 cc. of boiling water to which a few drops of HCl were added. The insoluble material was filtered off and discarded. $AgNO_3$ was now added to the filtrate until a test on samples gave a brown instead of yellow precipitate with $Ba(OH)_2$ solution. The

precipitated AgCl was filtered off. NaOH solution was then carefully added. At a pH of 6.0 a brown, slimy precipitate was formed. This was filtered off and the filtrate saturated with $Ba(OH)_2$. The black precipitate thus formed was filtered off and washed thoroughly with hot water. It was suspended in 500 cc. of water and dilute HCl was added until the reaction was acid. The AgCl was filtered off. It was necessary to extract with dilute acid several times to separate all the guanidine compound. The aqueous filtrates were evaporated to dryness on the water bath. The residue was extracted with absolute alcohol to remove inorganic salts. This alcohol extract was taken for preparing the double salts of the guanidine derivative used in its identification.

TABLE II.

Melting Points of Homologous Diguanidino Polymethylenes.

Diguanidino compound.	Picrate.	Chloro-aurate.	Chloro-platinate.	Sulfate.
	°C.	°C.	°C.	°C.
Ethylene		258	258	290
Trimethylene	242	184	246	300 (Over.)
Pentamethylene	226	161		
Octamethylene	206	143	216	286
Decamethylene (synthalin)	193	153	211	
Glukhorment base	191	152	209	
Neosynthalin	190	117	206	

The constants for the first three compounds are taken from the literature. The synthesis of diguanidino octamethylene will be reported in a later paper. Neosynthalin appears to be isomeric with diguanidino dodecamethylene and may be identical with it.

In order to obtain the hydrochloride in a pure, dry form, the alcoholic solution was evaporated to dryness, and the residue crystallized from 10 cc. of water. The material so obtained was used in the rabbit tests for obtaining the minimum effective dose.

Picrate.—The picrates of the synthalin and glukhorment guanidine bases were made by adding an excess of an aqueous solution of picric acid to a dilute alcohol-water solution of the hydrochlorides. The synthalin picrate melted at 191.5–193° uncorrected. The glukhorment picrate melted at 189–190° uncorrected. One recrystallization raised the melting point of the latter compound 1°. Equal amounts of the synthalin and the

glukhorment picrates were mixed with absolute alcohol. The solution was dried. The m.p. was 190–191.5°.

Chloroaurate.—The double gold salts of synthalin HCl and glukhorment HCl with $AuCl_3$ are quite soluble in alcohol. They were precipitated from aqueous solution and washed with water. The glukhorment double salt melted at 144–146° uncorrected. One crystallization from alcohol water raised the m.p. to 152° uncorrected. The synthalin chloroaurate melted at 153° uncorrected.

Chloroplatinates.—The chloroplatinates were precipitated in alcohol water solution of the hydrochlorides of the bases, washed with absolute alcohol, and dried at 115°. They both melted with decomposition in a very characteristic manner. About 2° below the melting point, the material blackens. At the melting point there is a vigorous frothing. The synthalin chloroplatinate melted at 211° uncorrected; the glukhorment compound 2° lower. The former analyzed 29.1 per cent Pt; the latter 29.4 per cent Pt. Nitrogen determinations were made by combustion, 12.3 per cent being obtained for synthalin chloroplatinate and 12.7 per cent for glukhorment. It seems opportune, at this point, to comment upon our experience in determining the nitrogen content of guanidine compounds. For the platinum compound of synthalin, we have found it impossible to use the Kjeldahl method. Typical values obtained were 8.2 per cent by micro Kjeldahl and 10.5 per cent by macro Kjeldahl method with the $HgSO_4$ modification. In the cases cited, the Kjeldahl values checked with triplicate samples. The value of 12.3 per cent by combustion checks very closely the theoretical value of diguanyl diamino decane chloroplatinate (12.5 per cent).

BIBLIOGRAPHY.

1. von Noorden, C., *Klin. Woch.*, 1927, vi, 1041.
2. Blatherwick, N. R., Sahyun, M., and Hill, E., *J. Biol. Chem.*, 1927, lxxv, 671.
3. Bennett, T. I., *Lancet*, 1927, ccxiii, 519.
4. Fränkel, S., and Friedmann, M., *Biochem. Z.*, 1927, clxxxii, 434.
5. Sakaguchi, S., *J. Biochem.*, Japan, 1925, v, 25.

POLARIZED LIGHT AND STARCH HYDROLYSIS.

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The papers of Semmens (1, 2) and Baly and Semmens (3) indicate that polarized light exerts a selective action in accelerating hydrolysis of starch with diastase. This work was criticized by Jones (4) whose criticism brought forth a defense by Baly (5). The latter quotes from a brief note of Macht (6) which seemed to substantiate the original findings. Jones was unable to repeat the observations in his own laboratory.

Other papers by Bryant (7), Morrison (8), Bhatmagar and Lal (9), and Macht (10) indicate that polarized light has some effect on bacterial growth. Various reports from the laboratory of Macht deal with polarized light and toxicological effects of drugs, on metabolism in experimental avitaminosis, *etc.*, and strengthen the interest in the subject, although they have no bearing upon the particular question of starch hydrolysis.

Examination of the literature supplemented by correspondence with authors concerning details of experiments failed to convince us that adequate accuracy of technique had been maintained in all cases to justify the conclusions derived. Without adequate checks of light intensity, temperature, and quantitative analytical technique, in addition to measurements of polarization of light source, no conclusions one way or the other are permissible.

In view of the increasing volume of reports upon polarized light and its biological effects, it seems advisable to determine definitely whether under accurately controlled conditions such effects can be repeatedly secured. The conflicting reports suggest that some other factor than polarization of illumination may have been responsible for the positive findings attributed thereto.

In view of what has already been done and in light of prelimi-

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nary experiments in this laboratory by Stevens and Bunker[1] it seemed advisable to begin by attempting to repeat if possible the work of others on starch hydrolysis, and then, by rigidly controlling all possible variables, to determine whether positive results were actually due to polarized light or to some other factor, associated with or influenced by the polarization of light.

As a result of this work, we are forced to the conclusion that our results support the view that polarized light has no proved effect upon starch conversion by diastase. This conclusion, being a negative finding, in direct opposition to the publications of Baly and Semmens *et al.* can be of value only in light of the judgment as to whether our technique was adequate or faulty. This technique is given in detail in this paper for two additional reasons: first, in order that others interested may duplicate exactly the steps taken, and second, because we hope it serves in some degree as an example of an attempt to be scientifically accurate in the physics and chemistry of a biological investigation.

General Procedure.

The general procedure was to expose simultaneously similar aliquots of an identical preparation of starch and enzyme under standardized temperature conditions to illumination by (1) plane-polarized light, (2) heterogeneous light from the same source and of same intensity, (3) control in darkness.

To secure uniformity of temperature, all digestion work was carried out in an air bath constructed of wood and cork board with suitable doors for sampling. The bath was electrically heated under thermostatic control and the variations in temperature at one spot in the line of air travel inside the bath were during a run of not more than $\pm$ 0.02°, measured on a Beckmann thermometer. The air temperature probably varied more than this owing to the lag of the mercury thermometer, but on the other hand, the reaction mixtures of greater volumes and specific heats than the mercury in the thermometer may be assumed to have varied less than this amount.

The temperature of digestion in most experiments was approximately 40°.

[1] Unpublished data.

Empty cells were placed in the air bath to bring them to the proper temperature at least an hour before using.

Mixtures of buffer and starch were brought to temperature independently of the enzyme solutions and then mixed. After

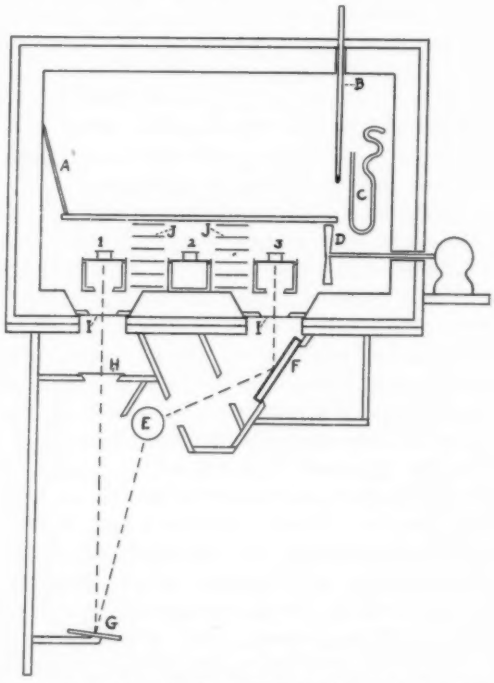

FIG. 1. Air bath and method of illumination. *A*, heating element; *B*, Beckmann thermometer; *C*, thermoregulator; *D*, fan; *E*, 200 watt Mazda lamp; *F*, pile of eight pieces of Bavarian plate glass; *G*, silvered plate glass mirror; *H*, single piece of Bavarian plate glass; *I*, *I*, windows of plate glass; *J*, *J*, light shields; 1, 2, 3, cells.

thorough agitation, aliquots were measured into the respective cells. At first, toluene was added to each cell as a precaution against bacterial interference. This practice was later abandoned since it introduced errors in sampling the products of hydrolysis, by interfering with proper draining of pipettes.

The degree of hydrolysis in each cell was determined upon samples taken from time to time, either by a determination of reducing sugar produced, or by comparing the color with iodine against a double standard in a specially designed bicolorimeter.

The arrangement of air bath and the provisions for irradiating the cells are shown in Fig. 1.

The bath was built of wood and lined with 2 inch cork board. Hand holes in the large door permitted filling and withdrawal of samples from the cells without removing them from the bath or disturbing the temperature of the air in the bath. The cells used were made of rings of glass cemented to a slide, or of bottle sections attached to glass with De Khotinsky cement.

As Nicol prisms of suitable size were not available, glass plates in a pile set at a polarizing angle were employed. Even lantern slide cover-glasses were found to be not sufficiently plane, and Bavarian plate $4'' \times 6'' \times \frac{1}{8}''$ was used. A pile of eight plates at the proper angle gave a beam of five foot-candles intensity, highly plane-polarized as examined with a crossed Nicol.

The heterogeneous beam, reflected from a silvered mirror, was freed from excessive polarization in one plane by the inclined plate *H*. The intensity of this beam was made to match the other, as determined by the Macbeth illuminometer.

Non-reflecting screens cut off stray light.

Assuming that the effects of polarized light are pronounced, as indicated by the literature, several short runs were made with starch, taka-diastase, and buffered solutions, the effect being measured with the Folin-Wu (11) method of sugar determination.

The buffered solutions were made from 0.33 M sodium dihydrogen phosphate and 0.33 M disodium hydrogen phosphate, each containing 0.25 mol of sodium chloride. Either of these or a mixture of the two, when added to four times its volume of distilled water, would furnish the concentration of phosphates and sodium chloride shown to be optimum for diastatic action of taka-diastase by Sherman, Thomas, and Baldwin (12). The H ion concentrations were measured by the drop method of Gillespie (13), checked against the hydrogen electrode.

Three attempts to hydrolyze raw starch from potatoes at pH 4.8 or 6.7 failed to show any rupturing of the grains, any reducing sugar, or color with IKI in the supernatant liquid after centrifug-

ing. This is in accord with similar observations by Bunker and Stevens[1] in 1925.

Starch grains, swelled with water at a temperature between 60–70° could be hydrolyzed. Of twenty-two determinations all showed hydrolysis but the differences were too small to be significant; likewise in eight runs with barley starch (warmed), starch from sprouted barley, and partially ripened bananas. Raw starch was not hydrolyzed if the precaution was taken to separate, by centrifuging, the whole grains from the ruptured grains before setting up the system. Ruptured grains are hydrolyzed.

Lest the conditions had been brought so near to optimum, or the reaction so stabilized that a slight additional energy increment from polarized light could not be demonstrated, buffers were abandoned for fourteen runs. Results with distilled water or tap water, without buffers, were so erratic that not only was it impossible to compare between the two kinds of illumination, but it was even impossible to duplicate results when water and reagents from a given set of stock bottles were used. This surprising result suggests that in a limited number of observations, one might be led to believe that polarized light, heterogeneous light, or darkness is superior to the other two conditions by as much as 57 per cent difference, if unbuffered solutions are used. It is only by numerous repetitions that the inherent error of such method is proved.

The foregoing experiments are reported, although without detail, because the conclusions regarding whole starch grains are at variance with preceding reports by others, and because the importance of buffers in such tests seems not to have been adequately stressed elsewhere.

In further work greater precision of measurement than that of the unmodified Folin-Wu method, and certain refinements of apparatus were employed.

Cells were prepared of 30 cc. capacity, stoppered with rubber stoppers fitted with valves to diminish evaporation, while slight expansion and contraction were allowed for. The bottoms of one pair of cells were cut from the same piece of plate glass.

Solutions for the cells were made from 4.5 cc. of 4 per cent suspension of potato starch added to 150 cc. of boiling phosphate-sodium chloride buffer of pH 6.2. After boiling 1 minute and

then cooling to the bath temperature, there was added 0.25 cc. of 0.25 per cent solution of taka-diastase in buffered solution. The cells previously held in the air bath 1 hour were filled from this mixture without removal from their respective positions, and the stoppers replaced at once.

Sugar determinations were made by the Folin-Wu method, modified for the following reasons. The amount of copper reduced by a given amount of glucose is influenced by the hydrogen ion concentration of the mixture in which the reduction takes place (14). Fig. 2 shows how the experimentally determined values of

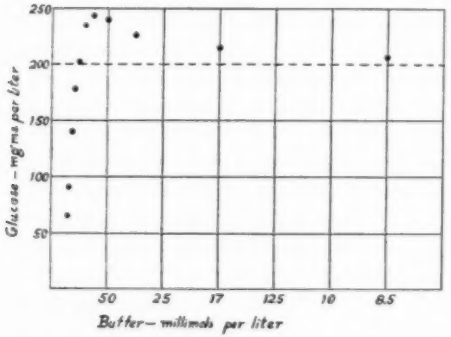

FIG. 2. Effect on glucose determinations caused by varying concentrations of buffer containing 1 part of Na_2HPO_4 + 99 parts of NaH_2PO_4. The true value is 200 mg. per liter. The determined values are indicated by dots.

reducing sugar differed from actual concentration when a poorly buffered standard was used, in measuring sugar solutions of varying buffer value. To secure accurate determinations it is necessary that unknown and standard both be of similar pH and buffer values. For our work, therefore, standards and starch solutions were prepared of the same buffer index. A second error occurs if the standard and unknown are of appreciably different sugar content. If the unknown is weaker than the standard, the result is lower than it should be; if stronger, the result read is too high. Wright (15) has shown that satisfactory corrections may be applied only if such corrections are individually determined for

each set of experiments. We found that with the reagents used in these experiments, the following corrections are fairly satisfactory.

Standard.	Correct reading.	
100 mg. per liter at 20 mm.	$R_{corr.} = R_{obs.} - \frac{R_{obs.} - 20}{4}$	(1)
200 " " " " 20 "	$R_{corr.} = R_{obs.} - \frac{R_{obs.} - 20}{10}$	(2)

In order to obtain more reliable results it is necessary to employ a standard whose color intensity is very nearly the same as that of the unknown. The preparation of enough standards to cover the range of values to be expected in any given run, with sufficiently small intervals of difference, was not practicable. The following method was devised to overcome this difficulty, and proved satisfactory.

Stock standards were prepared in concentrations of 400, 287, 178, 119, and 79 mg. of glucose per liter. (Each is $\frac{2}{3}$ the concentration of the next higher, and $1\frac{1}{2}$ times that of the next lower.) In the test of the method we employed concentrations for our unknowns which were the mean of each pair of adjacent stock standards; *i.e.*, 333, 222, 148, and 98.7. In our trial of the method the color was then developed on these unknowns in the usual way on 2 cc. portions of each, and the color compared with a working standard prepared by mixing equal portions of next higher and lower stock standards, after the color in each had been independently developed. It was assumed that the color value of the working standard so prepared was the mean value of the two stock standards from which it was prepared.

All results experimentally obtained in this test of the method, agreed with the true value of the unknowns. Each trial was repeated nine times, and the average errors were less than 0.5 per cent, irrespective of the concentration of sugar which was being measured.

The points chosen for the unknowns were purposely taken midway between the stock standards where the greatest error occurs in the usual method of unadjusted arbitrary standards. Since the determined error at this point is so small, it is assumed that the method is suitable for the entire range of color comparison.

Table I shows the proportions of any two adjacent stock standards to be mixed to yield any intermediate working standard.

In using the method, the concentration of sugar is first roughly determined by comparison with the stock standard which it most nearly matches, and by correcting the reading by Equation 1 or 2, interpolating or extrapolating where necessary, and solving the equation by the usual

$$C_u = \frac{C_{st} \times R_{st}}{R_u}$$

The concentration of the unknown (C_u), or a close approximation to it, is then found in Columns 1 to 6 of Table I. On the

TABLE I.

Showing Proportions in Which Standards Are to Be Mixed in Order to Make Desired Intermediate Standards.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
400	267	178	119	79	52.7	24	0
385	257	171	114	76.2	50.7	24	3
374	249	166	111	73.8	49.2	24	6
363	242	162	108	71.8	47.8	24	9
349	233	155	103	68.9	45.9	24	15
338	225	150	100	66.7	44.5	24	21
333	222	148	98.7	65.8	43.8	24	24
320	213	142	94.8	63.2	42.2	16	24
311	207	138	92.3	61.5	41	12	24
300	200	133	89	59.3	39.5	8	24
286	190	127	84.7	56.4	37.7	4	24

same line in Column 7 will be found the amount to be taken of the stock standard whose value heads the column in which the C_u was found. In Column 8 on the same line is the amount of the next lower stock standard to be mixed with it to furnish a useful working standard. A new comparison with this working standard is now made in the colorimeter.

Determinations of Conversion of Potato Starch to Reducing Sugar by Taka-Diastase.

Cells were set up with potato starch and taka-diastase in buffer solution, three at a time for four different runs each of 7 or 8 hours duration, at the temperature already specified.

Sugar determinations were made at time intervals as indicated as abscissæ in Fig. 3. The curves themselves are drawn through the points obtained by determining the concentration of reducing sugar in the cells held in the dark, but the individual dark determination points are omitted to make the diagram less complicated. Dark points were determined with the same frequency as the plotted light points. The curves are dropped each ten units below the preceding one and set over a few minutes to the right to avoid crowding.

Each point plotted represents a triplicate or in some cases a duplicate determination, the results and times of which are

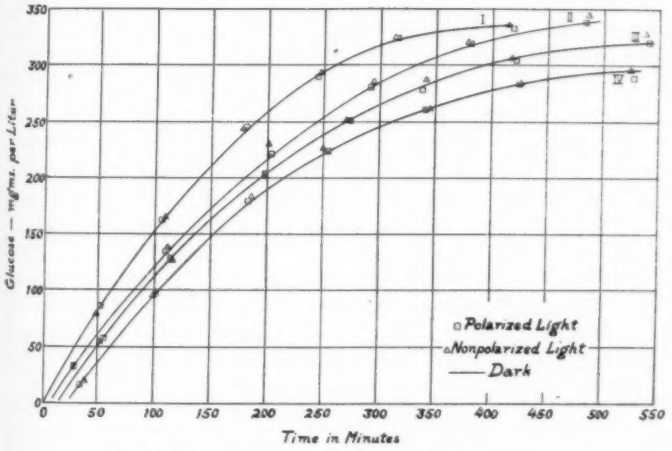

FIG. 3. Conversion of potato starch to glucose.

averaged on the working assumption that over such a short time interval (2 to 5 minutes) the curve could be regarded as a straight line.

Time was recorded as the number of minutes from the addition of enzyme to starch solution to the withdrawal of the sample from the cell. As each sample was discharged from the pipette into alkaline copper tartrate it was assumed that hydrolysis stopped.

Triplicate 2 cc. samples were taken from each cell as rapidly as possible. Previous work indicated that the expected hydrolysis

rate in each cell would be approximately the same. Three 2 cc. portions of stock standards which would probably cover the range of the three unknowns (taken at slightly different times) were

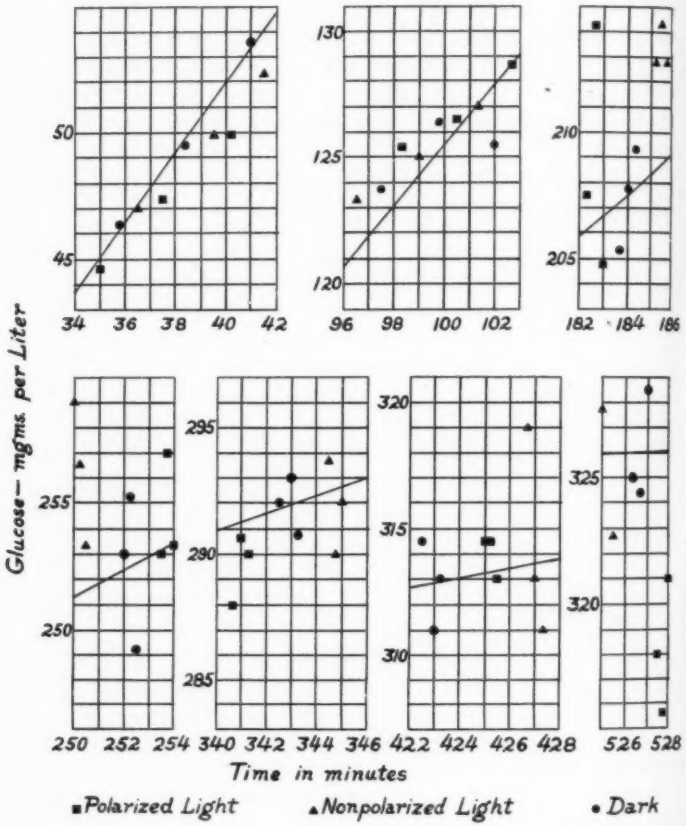

FIG. 4. Method of computing values for points plotted in Fig. 3.

selected. The one which most nearly matched the unknown taken in the mid position (in respect to time) was selected from which to prepare a working standard, from the data in Table I. From this the concentrations in the unknowns were determined.

In Fig. 4 are shown on a larger scale typical sets of individual determinations which furnished the average values plotted in Fig. 3.

It appears in Fig. 3 that hydrolysis is slightly more complete in each of four runs at the end of 7 or 8 hours in those cells which were exposed to heterogeneous light than in the corresponding cells exposed to plane-polarized light, but the differences are slight at every point in the curve.

It will be shown later that these differences are due not to the nature of the illumination but to a slight temperature gradient between cells even though the temperature of the bath was closely controlled. The amount of this temperature difference between cells is shown by thermocouple measurements to be not more than 0.37°. The air was circulated rapidly and continuously, but it is obvious that the air leaving the heater element was hottest and that the air just before it reached the heater again was coolest. The air around the first cell to be reached was accordingly warmer than that reaching the last cell in the course of air travel. In the experiments just reported it so happened that the warm air reached the cell illuminated by heterogeneous light first, the dark cell second, and that illuminated by polarized light third.

Conversion of Starch to Dextrins.

Macht (10) has reported that, as estimated by the color reaction with iodine, the hydrolysis of soluble starch in the presence of taka-diastase is accelerated by exposure to polarized light. This type of reaction was therefore studied. Except for changes made necessary by employing the IKI reaction, the technique was the same as that already recorded.

The concentration of starch was increased to 3.4 gm. per liter and the taka-diastase was decreased to 2 mg. per liter. The higher concentration of starch made it possible to make determinations on 1 cc. samples except in the later stages when, owing to absence of starch and low concentration of erythrodextrin, larger samples were taken. The lower concentration of enzyme gave a slower rate of conversion and permitted the reaction to be studied for 6 to 7 hours before the color with iodine became too faint to match.

Each sample as taken from the cell was added to 25 cc. of solu-

tion containing 0.03 gm. of iodine and 0.06 gm. of potassium iodide per liter. The color so obtained was sufficiently stable to warrant the assumption that hydrolysis was stopped by this mixing.

As a rule, a group of thirteen samples was taken at 2.5 minute intervals. Every other one of these (Samples 1, 3, 5, *etc.*) was taken from the control in the dark. The intervals between successive dark controls was therefore 5 minutes. Samples 2, 6, and 10 were from the cell in heterogeneous light; while Samples 4, 8, and 12 were from the cell in polarized light. The order of sampling, it will be evident, was dark, heterogeneous, dark, polarized, dark, *etc.*

Each sample from a lighted cell was calculated against two dark adjacent samples that nearly matched it in color. To accomplish this, a special colorimeter was built upon a Bausch and Lomb

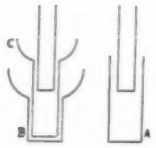

FIG. 5. Arrangement of cups in Anderson bicolorimeter.

biological colorimeter chassis. The scheme of cups is shown in Fig. 5. All three cups are independently adjustable by rack and pinion and each has its own vernier scale.

If one attempts to match the iodine color of partially hydrolyzed starch against a combination of superimposed colors developed from starch and erythrodextrin respectively, it will be found impossible to match the shade of the first solution with any combination of the two latter. This difficulty may be circumvented by using in Cups B and C, samples of the same hydrolysis as in Cup A but taken in point of time before and after the sample in Cup A. The unknown is now matched against these and the result expressed, not in absolute quantities, but as "equivalent time of hydrolysis," the progress of the dark hydrolysis being used as a basis of comparison. When all three rates of hydrolysis are substantially the same this proves a useful method of comparison.

In Fig. 6, if the ordinate scale of 100 is taken as the time of hydrolysis in the dark, the curve for the latter becomes a horizontal line, saving space and making it possible to plot the ordinates of the other hydrolyses on a larger scale and bring out more clearly any differences.

It was found that best results were obtained by using in Cups B and C (Fig. 5) solutions in which the hydrolysis time did not differ by more than 20 minutes.

The first seven of the thirteen samples were therefore matched against Samples 1 and 7, and the last seven samples against the

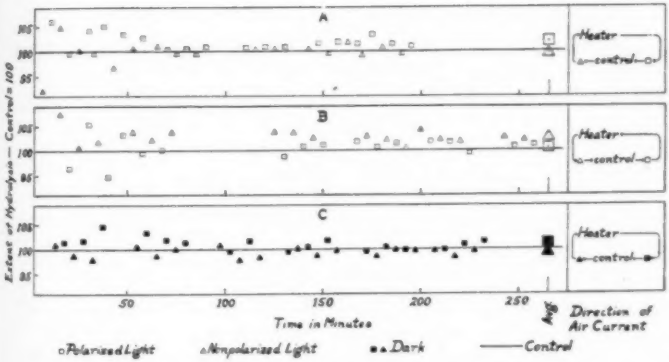

FIG. 6. Effect of reversing air current upon hydrolyses.

pair, Samples 7 and 13. Thus Sample 7 was matched against each pair of standards.

Each of the thirteen samples was matched in Cup A, set at the same level as Cup B, by varying the proportion of the color in BC through movement of Cup C. The readings of Cup C serve as a satisfactory record of differences between samples in Cup A, and furnished the data for the points plotted in Fig. 6.

Table II shows how these points were determined for the first two measurements of the sample in Cup A in Fig. 6.

The calculation from the colorimeter reading was done in the following manner. The first two controls (Samples 1 and 3, Table II) differed in hydrolysis by 5 minutes and by 7.2 mm. in their readings in the colorimeter. Each mm. therefore corresponded to $\frac{5}{7.2}$, or 0.7 minute. The first irradiated specimen

(Sample 2) differed from the first control by 3 mm.; that is, 2.1 minutes. The "equivalent time" is, therefore, 2.5 + 2.1 minutes = 4.6 minutes, or 92 on the scale of 100. By the same calculation the sample from the cell that had been kept in polarized light (Sample 4) may be shown to be 3.1 minutes ahead of Sample 3 in "equivalent time," giving a value of 10.6 minutes, or 106 on the scale of 100. The apparent magnitude of the errors or irregularities in the earlier determinations is due to their exaggeration on changing to a scale of 100. Actually, as measured in minutes, the irregularities were small.

TABLE II.

Computation of Comparative Hydrolysis Values for First Five Points Plotted on Fig. 6 A.

Cup A = Samples 1, 2, 3, *etc.* Cup B = Dark Sample 1. Cup C = Dark Sample 7.

Sample No.	Source.	True time.	Reading of Cup C.	Change in 5 min.	Equivalent time.	Comparative value.
		min.	*mm.*	*mm.*	*min.*	
1	Dark.	2.5	0		2.5	100
2	Non-polarized.	5.0	3		4.6	92
3	Dark.	7.5	7.2	7.2–0	7.5	100
3	Dark.	7.5	7.2		7.5	100
4	Polarized.	10.0	11.8		10.6	106
5	Dark.	12.5	14.7	14.7–7.2	12.5	100

The method of computing Samples 2 and 4 is given in the text.

The results of five runs each are recorded in A and B of Fig. 6. In a single run the time required for comparing in the colorimeter the solutions from a group of thirteen samples necessarily left a blank space in the record between the values for this group and the next. The determinations in the different runs were therefore timed so as partially to fill in these portions. The results of all determinations were recorded; none was thrown out because of failure to agree with the majority.

The results are averaged in the values plotted in Fig. 6 as large squares and triangles, and at the extreme right is shown the direction of the air current in each case. The results plotted in

C were obtained with all three cells in the dark, but in the same positions as in the regular runs. That is to say, the only difference was that the light was off during the two runs that furnished the data for C. The results show that in none of our experiments did polarized light have any special effect on the rate of hydrolysis. It is true that the reaction proceeded somewhat faster in light than in the dark, but the same result was observed whether the solution was exposed to heterogeneous or polarized light, and was most probably caused by the slight increase in temperature due to radiations absorbed at the lower surfaces of the rubber stoppers with which the cells were covered. The apparent differences in the effects of polarized and non-polarized light were apparently due entirely to differences in temperature. By reversing the direction of the air current, and with it the slope of the temperature gradient, either of the two irradiated specimens could be made to exceed the other in rate of hydrolysis.

SUMMARY AND CONCLUSIONS.

Numerous experiments have been made to determine any effect of polarized light on the hydrolysis of starch by taka-diastase. Starch grains, swelled starch grains, broken starch, and soluble starch have been employed. Starch from potato, barley, and banana has been studied at various times. Raw starch from sprouted barley with its own enzymes instead of taka-diastase was included.

Measurements of starch hydrolysis were made by the Folin-Wu method of determining blood sugar, and by a modification of this method employing solutions throughout of equal buffer index. Measurements of hydrolysis were also made with a specially designed bicolorimeter, in which hydrolyses more or less advanced than the unknown were employed and the combined starch-dextrin color components measured by the superimposed standards.

Although the temperature of the air bath was at all times constant within $\pm 0.02°$ at a given point, and although there was a rapid circulation of air at all times, there was nevertheless a temperature gradient of 0.37° difference between the two cells illuminated respectively with polarized and with non-polarized light. This temperature difference caused a variation in hydrolysis after several hours.

By changing the direction of the air current, it was possible to demonstrate beyond doubt that in these experiments there was no accelerating effect of polarized light upon starch hydrolysis by diastase.

These results are in disagreement with the original reports upon the subject and with two reported confirmations of the original reports, although agreeing with the one reported failure to confirm them. Being unable to gain access to sufficiently exact data on any previous work, and being entirely unaware of the precision of measurements upon which earlier conclusions were based, we do not know whether conditions in our experiments were comparable to those in earlier work. In order that others interested in the subject may repeat this work, if they desire, we have given complete essential data on all experiments.

In the absence of such data in other reports, and in light of the subtle nature of the errors which creep into measurements of enzyme activity (some of which we have indicated), we feel that the burden of proof lies with those who believe that polarized light, rather than some unchecked error of manipulation or observation, is responsible for the accelerated hydrolyses reported by them.

BIBLIOGRAPHY.

1. Semmens, E. S., *Nature*, 1923, cxi, 49.
2. Semmens, E. S., *J. Soc. Chem. Ind.*, 1923, xlii, 716.
3. Baly, E. C. C., and Semmens, E. S., *Proc. Roy. Soc. London, Series B*, 1924, xcvii, 250.
4. Jones, W. N., *Ann. Bot.*, 1925, xxxix, 651.
5. Baly, E. C. C., and Semmens, E. S., *Nature*, 1925, cxvi, 817.
6. Macht, D. I., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 472.
7. Bryant, E. G., *J. Soc. Chem. Ind.*, 1923, xlii, 681.
8. Morrison, T. F., *Science*, 1925, lxi, 392.
9. Bhatmagar, S. S., and Lal, R. B., *Nature*, 1926, cxvii, 302.
10. Macht, D. I., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 473.
11. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
12. Sherman, H. C., Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 231.
13. Gillespie, L. J., *J. Am. Chem. Soc.*, 1920, xlii, 742.
14. Folin, O., *J. Biol. Chem.*, 1926, lxvii, 357. Visscher, M. B., *J. Biol. Chem.*, 1926, lxix, 1. Somogyi, M., *J. Biol. Chem.*, 1926, lxx, 599.
15. Wright, S. L., Jr., *J. Biol. Chem.*, 1926, lxxi, 209.

SOME CHEMICAL INVESTIGATIONS OF EMBRYONIC METABOLISM.

I. THE ISOLATION OF FOUR PENTOSE NUCLEOTIDES FROM CHICKEN EMBRYOS.*

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INTRODUCTION.

A casual investigation of the literature may lead one to the conclusion that there is a hexose nucleic acid which occurs only in animal tissues and a pentose nucleic acid which is found only in plant tissues. While this conclusion may be correct, a disquieting factor presents itself in the wide-spread distribution of pentose nucleotides in animal tissues.

The first nucleic acid derivative of any kind to be isolated from animal tissues was inosinic acid, or hypoxanthine nucleotide, obtained from meat extract by Liebig in 1847 (1). Hammarsten (2) in 1894, isolated a β-nucleoprotein from the pancreas and found that it contained phosphoric acid, only one purine, namely guanine, and a substance which he believed was a pentose. From this nucleoprotein Steudel (3), in 1907, prepared guanylic acid, or guanine nucleotide, which has since been found in a large number of animal glands (4).

In 1914, Bass (5) reported the presence of purine bases in the blood in a combined form, one of which he identified as adenine and suggested that it is present in the form of adenylic acid. In 1923, Jackson (6) prepared a uranyl compound from human blood which had all the properties one would expect of the uranyl salt of adenine nucleotide. It contained adenine, gave the pentose color reactions, and contained its phosphoric acid in an easily hydrolyzable form. A year later (7) he obtained an amorphous substance

* The research reported in this paper has been greatly facilitated by the aid of a grant from the Faculty Research Fund of the University of Michigan for the purchase of a microanalytical balance and equipment. The author wishes to take this opportunity to express his appreciation to the committee in charge of this fund.

from blood and with the evidence furnished by his earlier work he concluded that he had "either the so called adenine uracil dinucleotide or what is far more likely, a mixture of nucleotides."

Adenine nucleotide was isolated in crystalline form from pig blood in 1925 by Hoffman (8). In the meantime Jones and Perkins (9), after preparation of large quantities of Hammarsten's β-nucleoprotein from the pancreas of both pig and beef, were able to isolate and definitely identify three pentose nucleotides from this substance. These were guanine nucleotide (guanylic acid), adenine nucleotide (adenylic acid), and cytosine nucleotide.

These pentose nucleotides may conceivably have their origin in the pentose nucleic acid of the diet, but their wide-spread distribution in animal tissues at least admits of the possibility of a doubt.

In 1885, Tichomiroff (10) showed that there was a synthesis of purine bases during the development of insect eggs, while a year later Kossel (11) showed that large quantities of purines appeared in the hen's egg during development, whereas no purine bases were present in the fresh egg. In 1903, Mesernitzki (12) investigated the purine content of hen's eggs before incubation and at different stages of development. He reported the purine bases as xanthine only and found that there was no increase. His method of hydrolysis would undoubtedly convert guanine into xanthine and adenine into hypoxanthine for he hydrolyzed the material by boiling with 5 per cent sulfuric acid for 15 hours. His results are probably not very significant since they do not agree with those of other investigators and his methods are doubtful. The work of Mendel and Leavenworth (13) agrees very closely with that of Kossel. Plimmer and Scott (14) have reported small amounts of nuclear material in the fresh egg and a very marked increase during the period of development. They did not isolate and identify any of the purine bases.

These investigations leave no doubt that purine bases are synthesized during embryonic development. Are they combined or free? If combined, are they present as hexose nucleic acid, pentose nucleic acid, or their derivatives? An attempt has been made in the present investigation to answer, at least, a part of these questions.

It is very difficult to isolate and purify pentose nucleic acid so that it is of constant composition and will lend itself to chemical analysis. However, the nucleotides which are obtained on hydrolysis of pentose nucleic acid can be purified by crystallization and have definite well defined properties. The methods used in this investigation would not yield pentose nucleic acid itself, even if present, but would enable one to isolate its hydrolysis products as nucleotides.

EXPERIMENTAL.

A preliminary investigation was made to determine whether or not chicken embryos would yield a β-nucleoprotein. Each embryo at the 18th day was found to contain about 350 mg. of what has been designated in this investigation as crude β-nucleoprotein.

After the preliminary investigation White Leghorn eggs were furnished by a hatchery during the months of February, March, and April, after they had been incubated for 18 days. A total of 412 embryos has been used, whose aggregate weight, including the shell, was 27.5 kilos, and which gave 153 gm. of crude β-nucleoprotein.

Crude β-Nucleoprotein.—The method is essentially the same as that followed by Jones and Perkins (9) in the isolation of nucleotides from the β-nucleoprotein of the pancreas of both pig and beef. However, it will be given in detail for the method used here gives uracil nucleotide also. A typical experiment will illustrate the procedure. The egg shells were opened and if the embryo was living the whole egg was ground, in a meat grinder, to a fine pulp. 127 embryos were treated in this manner and 5390 gm. of a semi-liquid pulp were obtained. The pulp was stirred with an equal weight of water for 1 hour, rapidly heated to boiling, and boiled for 10 minutes with constant stirring. It was then cooled by an outside freezing temperature or in an ice bath to room temperature or below, strained through four folds of cheese-cloth, and pressed out thoroughly. After two washings with 2 liters of water at each washing the milky extracts were combined and poured on folded filters. After filtering for 10 minutes the filtrates were water-clear. The filtrates of the first 10 minutes were refiltered. The filtration is extremely slow but can be allowed to proceed overnight without fear of decomposition. Sometimes it was necessary to let them go for 48 hours but bacterial decomposition is very likely to occur in that length of time, especially in a warm room. The β-nucleoprotein is not coagulable by heat and is present in the filtrate.

The filtrate was treated with glacial acetic acid to maximum turbidity (8 to 10 cc. per liter.) An equal volume of 95 per cent alcohol was then added and the precipitate allowed to settle overnight. The mother liquor was decanted, the residue centrifuged,

washed with absolute alcohol, centrifuged again, and dried in a vacuum desiccator over sulfuric acid.

Hydrolysis of β-Nucleoprotein.—The β-nucleoprotein was hydrolyzed with 20 per cent ammonium hydroxide, a method proposed by Calvery and Jones (15) for the hydrolysis of yeast nucleic acid. The method is slow, requiring 3 to 4 weeks, but has several advantages, as pointed out by the above authors. Since the effect of protein and primary protein decomposition products on the separation of the nucleotides into the adenine and guanine fractions was not known, it was decided to take the hydrolysate through the process of precipitation with lead acetate and the subsequent removal of the lead with hydrogen sulfide, before the usual separation into two fractions. The greater part of the protein material no doubt remained with the lead sulfide on the filter. The filtrate from the lead sulfide was aerated and evaporated to a small volume at a low temperature, then to a syrup in a vacuum desiccator, and finally hardened by grinding to a white powder with absolute alcohol. The nucleotides are insoluble in strong alcohol, whereas phosphoric acid, which is the chief impurity at this point, is readily soluble. The alcoholic filtrate was evaporated to a syrup and rehardened with absolute alcohol. In this way there is minimal loss of uracil nucleotide, the one most soluble in alcohol.

Adenine and Guanine Fractions.—The above hardened material was dissolved in 4 times its weight of hot water, made slightly alkaline with ammonium hydroxide, cooled, and an equal volume of absolute alcohol was added. This separates the nucleotides rather sharply into two fractions. The insoluble part consists almost wholly of the ammonium salt of guanine nucleotide and the soluble part contains the ammonium salts of the other three pentose nucleotides when they are present. Each fraction may be further purified by repeating the process.

Guanine Nucleotide.—Crystalline guanine nucleotide was prepared exactly as described by Buell and Perkins (16) and the details need not be repeated here. On analysis the following values were obtained:

Micro-Dumas-Pregl for nitrogen (17).

I. 3.287 mg. gave 0.510 cc. of N at 24° and 743 mm.
II. 3.438 " " 0.533 " " " " 23.5° " 743 "

Required for guanine nucleotide	N 17.54.
Found. I	" 17.46.
II	" 17.44.

Brucine Salts of the Adenine Fraction.—The filtrate from the ammonium salt of guanine nucleotide above was completely freed from guanine nucleotide and acidified with acetic acid. An equal volume of hot water was added and the material was taken through the lead procedure for the preparation of the free nucleotides. The filtrate from the lead sulfide was aerated, concentrated to a syrup, and hardened by grinding with absolute alcohol until the substance became a white powder. The alcoholic filtrate was evaporated to a syrup which was hardened by grinding with absolute alcohol a second time. The hardened material was dissolved in 3 times its weight of hot water and the solution

TABLE I.

Recrystallization No.	Per cent of N.
1	8.35
2	8.07
3	7.80
4	7.53
5	7.15
6	6.80
7	6.83
8	6.71
9	6.74
Required for brucine salt of uracil nucleotide	6.79

was neutralized to litmus with brucine. The brucine salts of the nucleotides were filtered and recrystallized nine times from 35 per cent alcohol.

Analysis of the brucine salts of the adenine fraction after each crystallization shows a diminishing nitrogen content and signifies a separation of the nucleotides by this process. Usually after the fifth or sixth crystallization the percentage of nitrogen is the same as that for the brucine salt of uracil nucleotide and does not change with subsequent recrystallizations. The percentage of nitrogen after each recrystallization is indicated in Table I.

Adenine Nucleotide.—The filtrates from the first and second crystallizations of the brucine salts contain most of the adenine nucleotide. The brucine salt of adenine nucleotide was carried

through the usual procedure for the preparation of the crystalline nucleotide. Analytical data are presented.

Micro-Dumas-Pregl for nitrogen (17).

I. 3.494 mg. gave 0.595 cc. of N at 24.5° and 742 mm.
II. 3.431 " " 0.585 " " " " 24.5° " 742 "

Required for adenine nucleotide	N 19.18.
Found. I	" 19.09.
II	" 19.13.

Cytosine Nucleotide.—The brucine salts obtained from the filtrates of the fourth, fifth, and sixth recrystallizations mentioned above were used for the preparation of cytosine nucleotide by the common procedure. The slightly high analytical values obtained at this point indicate that the cytosine nucleotide is practically free from even traces of adenine nucleotide as impurity.

Micro-Dumas-Pregl for nitrogen (17).

I. 3.743 mg. gave 0.440 cc. of N at 24.5° and 736 mm.
II. 4.115 " " 0.484 " " " " 24.5° " 736 "

Required for cytosine nucleotide	N 13.00.
Found. I	" 13.07.
II	" 13.08.

Uracil Nucleotide.—As the free nucleotide is very difficult to prepare in a crystalline form its preparation was not attempted with the small amount of material available. However, the crystalline ammonium salt was prepared, from which the crystalline lead salt was prepared according to the method described by Levene (18).

Micro-Dumas-Pregl for nitrogen (17).

I. 7.770 mg. gave 0.370 cc. of N at 24° and 739 mm.
II. 7.916 " " 0.379 " " " " 23° " 739 "

Required for lead salt of uracil nucleotide	N 5.29.
Found. I	" 5.32.
II	" 5.37.

DISCUSSION.

The foregoing evidence shows that developing chicken embryos synthesize the pentose nucleotides. It is quite probable also that they have the power to synthesize the nucleic acid which yields these nucleotides on hydrolysis. The author believes that the β-nucleoprotein obtained in this work is a protein conjugated with

what is commonly called yeast nucleic acid. The presence in animal tissues of all four of the pentose nucleotides which are obtained on hydrolysis of yeast nucleic acid makes it seem wise to substitute some other term for the nucleic acid of plants. Since the terms thymus or animal nucleic acid and yeast or plant nucleic acid no longer possess their original significance, the author suggests that the terms hexose nucleic acid and pentose nucleic acid be substituted for them. The nomenclature would be uniform, as we would then have *pentose nucleic acid*, *pentose nucleotide*, and *pentose nucleoside*; also *hexose nucleic acid*, *hexose nucleotide*, and *hexose nucleoside*. Since all the known nucleic acids and their derivatives belong to these two groups it is not necessary to designate them by their source and complicate the nomenclature.

SUMMARY.

1. A β-nucleoprotein has been prepared from chicken embryos which is similar in many of its properties to the β-nucleoprotein obtained originally by Hammarsten from pancreas.

2. All four of the pentose nucleotides obtained on hydrolysis of the nucleic acid from yeast have been obtained from the β-nucleoprotein of chicken embryos.

3. A suggestion is made to substitute the two terms hexose nucleic acid and pentose nucleic acid for the many terms that are used at the present time to refer to these two substances. The nomenclature for nucleic acid, nucleotide, and nucleoside would then be uniform.

BIBLIOGRAPHY.

1. Liebig, J., *Ann. Chem.*, 1847, lxii, 317.
2. Hammarsten, O., *Z. physiol. Chem.*, 1894, xix, 19.
3. Steudel, H., *Z. physiol. Chem.*, 1907, liii, 539.
4. Jones, W., and Rowntree, L. G., *J. Biol. Chem.*, 1908, iv, 289. Levene, P. A., and Mandel, J. A., *Biochem. Z.*, 1908, x, 221.
5. Bass, R., *Arch. exp. Path. u. Pharmakol.*, 1914, lxxvi, 40.
6. Jackson, H., Jr., *J. Biol. Chem.*, 1923, lvii, 121.
7. Jackson, H., Jr., *J. Biol. Chem.*, 1924, lix, 529.
8. Hoffman, W. S., *J. Biol. Chem.*, 1925, lxiii, 675.
9. Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1924–25, lxii, 291.
10. Tichomiroff, A., *Z. physiol. Chem.*, 1885, ix, 518, 566.
11. Kossel, A., *Z. physiol. Chem.*, 1886, x, 248.

12. Mesernitzki, *Russ. Wratsch.*, 1903, No. 27, 1001.
13. Mendel, L. B., and Leavenworth, C. S., *Am. J. Physiol.*, 1908, xxi, 77.
14. Plimmer, R. H. A., and Scott, F. H., *J. Physiol.*, 1909, xxxviii, 247.
15. Calvery, H. O., and Jones, W., *J. Biol. Chem.*, 1927, lxxiii, 73.
16. Buell, M. V., and Perkins, M. E., *J. Biol. Chem.*, 1927, lxxii, 21.
17. Pregl, F., Quantitative organic microanalysis, translated by Fyleman, E., Philadelphia, 2nd edition, 1924.
18. Levene, P. A., *J. Biol. Chem.*, 1919, xl, 395.

SOME CHEMICAL INVESTIGATIONS OF EMBRYONIC METABOLISM.

II. THE ISOLATION OF A HEXOSE NUCLEIC ACID FROM CHICKEN EMBRYOS.*

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(Received for publication, March 2, 1928.)

INTRODUCTION.

In the preceding paper the isolation of four pentose nucleotides from chicken embryos was reported. It was possible to isolate the nucleotides since they have very characteristic properties and are easily purified and identified. The nucleic acid from which they are derived is not easily purified and has no characteristic properties except that on hydrolysis it yields the nucleotides. This is not true of the known hexose nucleic acid occurring in nature. However, the work of Levene (1) and a number of other investigators (2–6), points very definitely to the fact that the hexose nucleic acid is a hexose tetranucleotide similar in structure to the pentose nucleic acid. Yet the free hexose nucleotides have not been prepared.

The early work of Hammarsten (7) and the more recent methods (8) of preparation and purification of hexose nucleic acid from different sources make it possible for one to prepare, from the same animal tissues, the hexose nucleic acid and the pentose nucleotides, since there is a marked difference in the solubility in boiling water

* The research reported in this paper has been greatly facilitated by the aid of a grant from the Faculty Research Fund of the University of Michigan for the purchase of a microanalytical balance and equipment. The author wishes to take this opportunity to express his appreciation to the committee in charge of this fund.

of the α-nucleoprotein, which contains the hexose nucleic acid and the β-nucleoprotein which yields the pentose nucleotides. The isolation of a compound from chicken embryos, which has several of the properties of a hexose tetranucleotide and which the author believes to be identical with the hexose nucleic acid of other animal tissues, is reported below.

EXPERIMENTAL.

Hexose Nucleic Acid.—Since in the preceding investigation it has been shown possible to prepare pentose nucleotides from chicken embryos it seemed advisable to investigate the hexose nucleic acid content of the same material. It is well known that when tissues are ground to a pulp and suspended in boiling water the α-nucleoprotein is coagulated while the β-nucleoprotein remains in solution. Furthermore it is the α-nucleoprotein which contains the hexose nucleic acid and the β-nucleoprotein which contains the guanylic acid and other pentose nucleotides. A typical experiment is given.

Twenty whole embryos, at the age of 18 days, were ground to a fine pulp in a meat grinder. The total weight was 1011 gm. The pulp was stirred for 1 hour with an equal weight of cold water, heated rapidly to boiling, boiled with constant stirring for 10 minutes, and then cooled to about 15° in an ice bath. The resulting material was strained through four folds of cheese-cloth, pressed as dry as possible, washed twice with 300 cc. of water, and pressed out thoroughly each time.

The coagulum was added slowly to 1500 cc. of a boiling solution containing 24 gm. of sodium hydroxide and 75 gm. of sodium acetate, boiled for 10 minutes, and then heated on a steam bath for 2 hours. Most of the material dissolved. The resulting solution was diluted with 500 cc. of water and acetic acid was added until it showed a slight acidity to litmus. Care should be taken at this point. Adjustment of the acidity for most rapid filtration can be made by alternate addition of sodium hydroxide and acetic acid. About 35 cc. of glacial acetic acid are required. When the acidity was most favorable for rapid filtration the contents were heated to boiling and filtered on a hot water funnel. The filtrate was then evaporated to 300 cc. on a steam bath and the resulting concentrated solution, which was highly colored, was poured into a liter of

95 per cent alcohol. When allowed to stand overnight a flocculent precipitate, consisting chiefly of sodium nucleate, settled out. The brown-colored supernatant alcoholic solution was decanted and the precipitate washed twice by decantation with 95 per cent alcohol. The alcohol was pressed out as thoroughly as possible by means of a spatula. The precipitate was dissolved in 300 cc. of hot water, heated for 30 minutes on a steam bath, decanted from insoluble phosphates, and the decanted fluid treated with 10 cc. of 10 per cent sodium hydroxide. It was then filtered on a hot water funnel; the filtrate was made faintly acid with acetic acid and poured into a liter of 95 per cent alcohol. The precipitated sodium nucleate was washed twice by decantation with 95 per cent alcohol and twice with absolute alcohol, filtered, and dried in a vacuum desiccator. The yield was 2.5 gm. The material was further purified by the procedure described by Levene (1).

3 gm. of the above material were dissolved in 100 cc. of 0.5 per cent sodium hydroxide and boiled for 30 minutes. After neutralization with acetic acid, 10 cc. of 5 per cent colloidal iron were added. It was then filtered and 200 cc. of methyl alcohol containing 2 per cent of hydrochloric acid were added to the filtrate. The precipitate was washed with methyl alcohol until free from hydrochloric acid. After drying in a desiccator over sulfuric acid the yield was 1.2 gm.

From the method of preparation it is evident that this substance cannot be a pentose nucleic acid, for heating with alkali completely decomposes pentose nucleic acid, while hexose nucleic acid is not affected by this treatment ((8) p. 52), (1, 9–11). Steudel and his coworkers (9) have shown that treatment of pentose nucleic acid for only a few minutes at the room temperature with very dilute sodium hydroxide liberates the guanine nucleotide. Jones and Perkins (12, 13) realized the value of this method and have applied it extensively to the study of the hydrolysis products of pentose nucleic acid.

It may also be well to point out that there is other evidence against the possibility of this substance being a pentose nucleic acid. A 4 per cent solution of the hexose nucleic acid of other sources forms a gelatinous solution when cold (2) while this is not true of the pentose nucleic acid. The compound reported here forms a gelatinous solution in this concentration. Furthermore,

the characteristic pentose color reactions which are so strongly positive with yeast nucleic acid are not given by this compound.

Positive evidence is presented below in the form of analytical data, specific rotation (2), and the quantitative relationships of the adenine and guanine to the original substance and to each other.

Micro-Dumas-Pregl for nitrogen (14).

I. 6.850 mg. gave 0.878 cc. of N at 24° and 736 mm.
II. 5.123 " " 0.661 " " " " 25° " 736 "

	Calculated for hexose tetranucleotide.	Found.			
		I.	II.	III.	IV.
N	14.61	14.28	14.32		
P	8.62			8.83	8.91

III. 4.609 mg. gave 28.083 mg. of ammonium phosphomolybdate.
IV. 3.209 " " 19.634 " " " "

Specific Rotation.

Per cent.	Observed α.	$[\alpha]_D^{20}$
4.00	5.84	146.0
3.40	4.79	141.1
2.80	3.71	132.5
2.20	2.73	124.0
1.60	1.77	110.6
1.00	0.98	98.0
0.60	0.52	86.6
0.40	0.35	87.5

Preparation of Guanine.—400 mg. of the hexose nucleic acid were heated for 1 hour with 5 cc. of 7 per cent sulfuric acid on a steam bath. The solution, which was highly colored, was roughly neutralized, while still hot, with concentrated ammonia solution. An excess was then added until the solution contained about 2 per cent of ammonium hydroxide. When allowed to stand overnight the guanine precipitated almost quantitatively but usually contained a small amount of phosphates. The yield was 46 mg. or 11.50 per cent. The theoretical yield is 10.51 per cent. The crude guanine was dissolved in hot 5 per cent hydrochloric acid, decolorized with charcoal, and the colorless filtrate allowed to stand in an ice box. Guanine hydrochloride crystallized in beautiful needles.

Micro-Dumas-Pregl for nitrogen (14).

I. 2.677 mg. gave 0.757 cc. of N at 23° and 731 mm.
II. 2.304 " " 0.655 " " " " 24° " 731 "

Micro-Pregl for water of crystallization (14).

III. 8.323 mg. lost 1.328 mg. in a vacuum desiccator.

	Calculated for $C_5H_5N_5O$·HCl·2H_2O.	Found. I.	II.	III.
N	31.33	31.38	31.44	
H_2O	16.10			15.96

Preparation of Adenine Picrate.—An excess of silver nitrate and of ammonium hydroxide were added to the filtrate from the guanine of the above experiment. Care must be taken that too large an excess of ammonium hydroxide is not added since the silver salts of the purines are soluble in large excesses of that reagent. The precipitate of silver adenine was removed by filtration, washed, and suspended in hot water. The silver was removed by an addition of a slight excess of hydrochloric acid. The silver chloride was removed by filtration, washed, and an excess of a saturated solution of picric acid was added to the combined filtrate and washings. After a short time the precipitate of adenine picrate separated. It had the characteristic appearance of matted hair. The melting point after recrystallization was 296° when heated rapidly as advocated by Vickery and Leavenworth (15). The yield was 96 mg.

Micro-Dumas-Pregl for nitrogen (14).

I. 3.561 mg. gave 0.936 cc. of N at 25° and 741 mm.
II. 2.575 " " 0.676 " " " " 24° " 741 "

	Calculated for $C_5H_5N_5$·$C_6H_2(NO_2)_3OH$·H_2O.	Found. I.	II.
N	29.31	29.37	29.43

DISCUSSION.

The evidence presented here is not absolute proof that a hexose nucleic acid has been obtained which is identical with thymus nucleic acid or the hexose nucleic acid obtained from any of the other animal organs. A diligent search of the literature would reveal that there is no absolute proof that any two of the known hexose nucleic acids are identical. There is so much evidence, however, that they have the same structure that their origin is often undesignated.

Since all the hexose nucleic acids agree closely in their properties and chemical composition it seems very likely that they do have the same structure. Furthermore, they yield the same products on hydrolysis. The only disagreement with this statement comes from the work of Johnson and Coghill (16), who have reported the isolation of 5-methyl cytosine from tuberculinic acid. As these authors have pointed out, there has been great uncertainty as to which pyrimidines function as primary constituents in the nucleic acid molecule. The work of Johnson and Coghill supports Jones and Perkins (17) in their belief that uracil nucleotide is a secondary derivative of cytosine nucleotide in the hydrolysis of yeast nucleic acid. Calvery and Jones (13) were unable to confirm the findings of Jones and Perkins when a different method of hydrolysis was used. Johnson and Coghill were fortunate in that tuberculinic acid contains two pyrimidines, one of which does not yield the other when it is deaminized. Whereas with yeast nucleic acid this is not the case. Their work is very significant, especially when one is considering the chemical composition of the hexose nucleic acids of other sources.

Since the chief interest in the present investigation was to identify a hexose nucleic acid as distinct from the pentose derivatives also isolated, no effort has been made to identify the pyrimidines which occur in the compound reported here. Further investigations of embryonic metabolism are being made from a quantitative standpoint.

SUMMARY.

A compound has been prepared from chicken embryos which is similar in a number of its chemical properties and some of its hydrolysis products to the hexose nucleic acid obtained from other sources. These similarities justify the belief that it is the hexose nucleic acid which normally occurs in animal tissues.

BIBLIOGRAPHY.

1. Levene, P. A., *J. Biol. Chem.*, 1922, liii, 441.
2. Jones, W., *J. Biol. Chem.*, 1908–09, v, 1.
3. Steudel, H., *Z. physiol. Chem.*, 1908, lv, 407; lvi, 212.
4. Levene, P. A., *J. Am. Chem. Soc.*, 1900, xxii, 329.
5. Levene, P. A., and Mandel, J. A., *Z. physiol. Chem.*, 1906–07, l, 1.

6. Levene, P. A., and Mandel, J. A., *Biochem. Z.*, 1908, x, 215.
7. Hammarsten, O., *Z. physiol. Chem.*, 1894, ix, 19.
8. Jones, W., Nucleic acids, New York and London, 2nd edition, 1920, 103.
9. Steudel, H., and Peiser, E., *Z. physiol. Chem.*, 1919, cviii, 42; 1921, cxiv, 201; 1922, cxx, 292. Steudel, H., and Izumi, S., *Z. physiol. Chem.*, 1923, cxxxi, 159. Steudel, H., and Nakagawa, S., *Z. physiol. Chem.*, 1923, cxxviii, 129.
10. Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.
11. Calvery, H. O., *J. Biol. Chem.*, 1927, lxxii, 27.
12. Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1924–25, lxii, 291.
13. Calvery, H. O., and Jones, W., *J. Biol. Chem.*, 1927, lxxiii, 73.
14. Pregl, F., Quantitative organic microanalysis, translated by Fyleman, E., Philadelphia, 2nd edition, 1924.
15. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1925, lxiii, 579.
16. Johnson, T. B., and Coghill, R. D., *J. Am. Chem. Soc.*, 1925, xlvii, 2838.
17. Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1924–25, lxii, 557.

TITRIMETRIC QUINHYDRONE ELECTRODES.

A COMPARISON WITH THE HYDROGEN ELECTRODE FOR HYDRION CONCENTRATION DETERMINATIONS IN PLASMA, WHOLE BLOOD, AND OTHER BIOLOGICAL FLUIDS.

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The increased interest in hydrion concentration measurements has created a need for a simple method capable of reproducing the results obtained with the hydrogen electrode. Various modifications of the colorimetric method at first appeared to provide such a substitute, but recent studies have shown them to be erratic in many biological fluids. Among the other possibilities for a simplified method, the quinhydrone electrode seems to be the most promising because of the economies in time, material, and equipment which it makes possible.

Several investigators (1–7) have reported the use of the quinhydrone electrode for the determination of the pH of whole blood but have not given sufficient evidence concerning its accuracy. Others (8–12) have declared it to be unsuitable for such a purpose but have found it applicable to serum or plasma. Disagreement exists among the latter in regard to the size of the variation between the hydrogen and quinhydrone electrodes, and also as to the best method for overcoming the drifts encountered when the quinhydrone electrode is used in undiluted serum or plasma. Cullen and Biilmann (8) found that the results obtained by the two methods agreed if the pH (quinhydrone) was calculated from readings made within 2 or 3 minutes after the addition of the quinhydrone. However, Vellinger and Roche (11) concluded that the quinhydrone electrode gave low values and found it necessary to extrapolate their readings to zero time and to correct

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for the protein content of their material. In their recent paper Cullen and Earle (12) also report the use of extrapolation to zero from three successive readings at 30 second intervals. By this procedure they found that the quinhydrone electrode results were consistently 0.06 pH lower than those obtained with the hydrogen electrode in normal human serum. The difference was smaller and variable in two pathological sera. The work reported in the present paper was completed before that of Cullen and Earle had appeared, and it likewise deals with a comparison of the quinhydrone and hydrogen electrodes. However, the results obtained are in agreement with those of Cullen and Biilmann and differ from those of the later workers. A number of other body fluids representing wide differences in reaction, buffering capacity, and protein content have been included in the comparison of the two methods.

Of the methods available for the evaluation of quinhydrone electrode potentials, that described by Meeker and Oser (5) in which two electrodes are balanced titrimetrically is the most simple since it eliminates the potentiometer and its accessories. In addition it possesses certain theoretical advantages in that the secondary changes taking place in the test cell are to some extent paralleled by simultaneous changes in the titration cell so that errors caused by these changes are partially compensated. For similar reasons the salt error is probably reduced. This method accordingly was adopted with modifications enabling the use of smaller amounts of material and making possible a decrease in the time required for the determination.

Method.

Briefly, the method is to balance the potential produced by the presence of quinhydrone in the solution of unknown pH with that similarly produced in a buffer mixture whose composition is known but which may be varied by the addition of either of the buffer components. Since the potentials are proportional to the pH, zero deflection of a galvanometer in the circuit indicates that the hydrion concentrations are the same in both cells. The pH may then be calculated from the relative proportions of the constituents of the buffer mixture.

A test cell having a volume of approximately 1 cc. was designed

for the present study. It was enlarged at the open end so as to take conveniently a stopper carrying the electrode, stirring device, and salt bridge. Below the enlargement a constriction divided the cell and acted as a baffle during stirring, in this way decreasing the possibility of mixing the surface with that portion of the contents in which the electrode was suspended. A second stopper carrying a thermometer was used during the interval required to bring the cell to the desired temperature.

The titration cell consisted of a small beaker similarly equipped with an electrode, thermometer, and stirring rod. An agar-KCl salt bridge completed the circuit. Both cells were immersed in a water bath. Biilmann and Krarup (13) have shown that the temperature coefficient for the quinhydrone electrode is -0.00074 volt per degree. Since this is equivalent to about 0.015 pH, care was taken to keep both cells at the temperature required for the determination. Either gold or platinum electrodes could be used in any of the fluids examined provided the electrodes were tested for equivalence before each determination. The quinhydrone was prepared by the oxidation of hydroquinone by $FeCl_3$ according to the method of Biilmann and Lund (14). Four different preparations gave uniformly good results.

The following buffer systems were used: borate-boric acid, primary-secondary phosphates, and citric acid-sodium phosphate. The selection of the proper buffer system when the reaction of the material was not approximately known was made by preliminary tests with indicators. From the data given by Clark (15) large scale curves were constructed for each buffer pair in which percentage composition was plotted against pH. It was observed that at a given pH and temperature the red color characterizing the autooxidation of quinhydrone or its dissociation products seemed to appear more rapidly in the phosphate than in the borate mixtures. A more thorough study of this point is contemplated.

Procedure.

A measured volume (usually 10 cc.) of one of the buffer solutions of the pair selected was placed in the titration cell. The solution of unknown pH was run into the test cell, precautions being taken to reduce mixing and to fill the cell completely. The stopper

with the thermometer was then inserted without leaving any air space. Most of the required buffer was then added from the burette to the titration cell and was followed by the necessary excess of quinhydrone. When the desired temperature had been reached, the test cell electrode was moistened with distilled water, dipped into quinhydrone, and the stopper carrying it quickly substituted for the original stopper. The exchange required only a few seconds. Since the displacement of the second stopper was greater than the first, the greater part of that portion of the contents which had been exposed to air was forced out of the cell when the second stopper was inserted.

Immediately after the addition of the quinhydrone to the test cell the contents of both cells were stirred not longer than 2 or 3 seconds. Guided by the deflections of the galvanometer produced by closing the circuit with the tapping key, the necessary additions of buffer solution were made from the burette until zero deflection was reached. Following this the temperatures were taken again. From the burette reading the composition of the buffer mixture in the titration cell was calculated and expressed as volumes per cent of one of the buffer components. This value being used, the pH was read from the buffer curves.

After equilibrium was reached, which in all cases required less than 15 seconds, the drift in human serum or plasma for approximately 1 minute was less than 0.01 pH. The data obtained within this time agreed well with the hydrogen electrode. Readings made after this interval became progressively more acid. With the procedure described the determinations may be completed easily within 1 minute.

The loss of CO_2 was minimized by keeping the material tightly stoppered and without air space in the cell. Only a small surface was exposed for a brief interval during the change of stoppers necessitated by the addition of quinhydrone, and most of that portion of the contents so exposed was forced from the cell when the stopper carrying the electrode was inserted in the cell. While this procedure was objectionable because of the possibilities for gas exchange, the design of the cell to a large extent prevented the mixing of that portion of the fluid below the baffle with that above so that the short exposure of the surface probably did not affect the determination. The excellent agreement between the hydrogen

TABLE I.

Comparative Results for pH of Human Plasma at 20°.

Date.	Patient.	Hydrogen electrode.		Quinhydrone electrode.		Difference.	Time.
		pH read.	Average.	pH read.	Average.		
							sec.
Mar. 21	Mo. (diabetes).	7.56	7.57	7.52	7.53	−0.04	
		7.57		7.54			
				7.54			
Apr. 1	De., serum (alcoholism).	7.62	7.63	7.60	7.63	0.00	40
		7.64		7.64			300
				7.64			10
				7.65			
" 6	Jo. (alcoholism).	7.58	7.59	7.58	7.59	0.00	20
		7.59		7.59			60
				7.59			80
" 13	Mixed plasma, two patients.	7.54	7.55	7.53	7.54	−0.01	40
		7.55		7.54			55
				7.56			21
" 8	Mixed plasma, equilibrated (normal).	7.32	7.33	7.32	7.33	0.00	
		7.33		7.33			
" 27	" "	7.57	7.58	7.57	7.58	0.00	45
		7.59		7.59			33
" 28	Re. (normal).	7.58	7.59	7.57	7.58	−0.01	48
		7.59		7.58			40
				7.58			50
May 2	Mixed plasma,* several days old.	6.16	6.16	6.15	6.17	+0.01	
				6.19			
" 4	Al. (acute alcoholism).	7.61	7.61	7.60	7.62	+0.01	30
		7.61		7.62			25
				7.63			35
				7.63			23
" 16	Dt. (delerium tremens).	7.69	7.69	7.69	7.71	+0.02	27
				7.70			28
				7.71			60
				7.73			40
June 16	Ja. (nephritis).	7.56	7.57	7.61	7.61	+0.04	25
		7.58		7.61			95

Average difference, signs considered	+0.001
" deviation (single quinhydrone electrode determinations from H electrode average)	0.017
Largest single deviations	±0.050

* Omitted from calculation of averages and deviations.

and quinhydrone electrodes confirmed this conclusion. Mineral oil cannot be used because of the difficulty of adding the quinhydrone through an oily layer.

Hydrogen electrode determinations were made with the apparatus and technique described by Cullen (16). The apparatus was periodically standardized against 0.1 N HCl, and before each determination against a phosphate buffer solution having a pH near that of the unknown fluid. The quinhydrone electrode was similarly controlled by determinations made with buffer solutions of known pH.

The study of dog serum was made with the material at 38°. For the other fluids, the two methods were compared at room temperature. The results should not lose their significance because of this fact since it is the comparison of the two methods under similar conditions with which this paper is concerned. Obviously, the data are not to be taken as an expression of the pH of the material at body temperature. The effects of increasing the temperature on the determination will be discussed under the experimental part.

With the exception of Sample Re. (Table I), the human blood was obtained from patients in the alcoholic wards of the Philadelphia General Hospital in connection with studies of the acid-base equilibrium of these patients.

EXPERIMENTAL.

Serum and Plasma.—When the quinhydrone electrode was used for pH determinations in human serum and plasma at 20°, the results were identical with those given by the hydrogen electrode within the limit of error of the two methods. No systematic difference was observed between normal and pathological samples which was in any way comparable to that reported by Cullen and Earle. Furthermore, the reproducibility of the quinhydrone electrode was practically as good as that of the hydrogen electrode.

Autooxidative changes in the serum became evident 15 to 30 seconds after mixing the quinhydrone with the solution, when the contents of the cell began to assume a pink tint. The time of appearance and the rate of increase of the red color seemed to be determined chiefly by the degree of alkalinity. However, other factors not related to the reaction complicated the secondary

decompositions so that the magnitude of the drifts caused by these changes could not be predicted. After the drifting had proceeded for some time and the solution had become dark red or brown the results were considerably more acid than the original values.

Fortunately the autooxidation proceeded slowly enough to enable the initial potential of the hydroquinone-quinone system governed by the pH to be estimated. Furthermore, the changes in potential which followed were roughly paralleled by the increased redness of the solution so that it was possible to judge approximately the validity of any determination from the color of the solution at the time the reading was made. Cullen and Biilmann (8) have pointed out that the readings must be made before a decidedly red coloration appears. The drifts encountered were not sufficiently constant to permit an accurate extrapolation to zero time. A series of determinations at 38° did not show any markedly accelerated drift. While the rate of change was perceptibly increased, the acceleration was not of sufficient magnitude to cause difficulty.

A number of comparisons with the Cullen colorimetric method with human plasma at 20°, corrected to 38° by subtracting 0.23, showed that values given by the quinhydrone electrode at 38° were consistently 0.01 to 0.08 pH lower than those obtained colorimetrically. Such a difference is to be expected since the tendency of the colorimetric method is to err in the direction of values more alkaline than those given by the hydrogen electrode. When compared with the colorimetric method and the hydrogen electrode at 20°, the quinhydrone electrode likewise gave results closer to those obtained by the hydrogen electrode than did the colorimetric method. In eight specimens of pathological human plasma the quinhydrone electrode deviations from the hydrogen electrode values varied from zero to 0.05 pH, averaging 0.016 pH. On the same specimens the colorimetric method corrected for temperature but not for dilution, varied between 0.03 and 0.14 pH with an average deviation of 0.075 pH.

Through the cooperation of Dr. D. Wright Wilson and Dr. C. G. Johnston of the Department of Physiological Chemistry of the University of Pennsylvania it was possible to obtain sera from dogs being used in the study of severe hemorrhage and

also to use the results of their determinations by the hydrogen electrode.

When used in dog serum, the quinhydrone electrode gave

TABLE II.

*Comparative Results for pH of Dog Serum at 38°.**

Date.		Hydrogen electrode.		Quinhydrone electrode.		Difference.	Time.
		pH read.	Average.	pH read.	Average.		
							sec.
May 5	Normal.	7.30	7.31	7.32	7.32	+0.01	60
		7.31		7.32			70
" 17	"	7.37	7.37	7.27	7.28	−0.09	65
		7.37		7.29			40
" 19	"	7.39	7.39	7.41	7.42	+0.03	
		7.39		7.42			45
June 22	"	7.35	7.36	7.33	7.33	−0.03	
		7.37					
" 29	"	7.32	7.32	7.32	7.34	+0.02	
		7.32		7.35			
" 22	After hemorrhage.	7.45	7.46	7.41	7.42	−0.04	20
		7.46		7.42			10
" 29	" "	7.27	7.27	7.32	7.32	+0.05	30
				7.32			35
" 29	" "	7.22	7.22	7.22	7.25	+0.03	60
				7.27			
" 30	" "	7.43	7.43	7.48	7.51	+0.08	32
				7.52			28
				7.53			5
" 30	" "	7.10	7.10	7.07	7.09	−0.01	30
	premortal.			7.11			25

Average difference, signs considered	+0.001
" deviation (single quinhydrone electrode determinations from H electrode average)	0.040
Largest single deviations	±0.100

* Hydrogen electrode determinations were taken from studies on acid-base equilibrium in severe hemorrhage by Dr. D. Wright Wilson and Dr. C. G. Johnston.

less satisfactory results than it did in human serum (Table II). Although most of the determinations showed fairly close agreement with the hydrogen electrode, the use of the

quinhydrone electrode in this material is not advisable when accurate results are desired, because of the possibility for the occurrence of deviations as large as ± 0.1 pH. Both normal sera and those obtained after severe hemorrhages seemed to be subject to such variations. While no marked increase in the rate of drift was observed in the samples exhibiting the greatest deviations, in general the drifts were more rapid and of greater magnitude than in human sera at the same temperature. Undoubtedly the increased deviations encountered in dog sera can be ascribed to a considerable extent to this acceleration of the change in the potential. It is significant in this connection that colorimetric determinations of the pH of dog serum are likewise less satisfactory than those of human serum. Cullen and Earle (12) state that the drift and reproducibility of the quinhydrone electrode in dog serum are apparently the same as in human serum, but that the difference between the quinhydrone and hydrogen electrodes is 0.09 pH instead of 0.06 pH which was found for human serum. Their comparisons were made at 20°.

The inability of the quinhydrone electrode to measure accurately the pH of solutions of more than slight alkalinity is perhaps its most important limitation. In several experiments the quinhydrone electrode was compared with the hydrogen electrode at reactions above those commonly encountered in life. The material used was horse serum equilibrated with known CO_2 mixtures. At pH 7.9 to 8.0 the quinhydrone results were within the limit of error of the hydrogen electrode determinations. However at a pH of 8.4, the change in potential was sufficiently rapid to make the evaluation of a null point practically impossible.

Whole Blood.—Our experience with whole blood was similar to that of Cullen and Biilmann (8). The quinhydrone appeared to react with the hemoglobin and as a result the readings were too high and the results were not reproducible. Whole blood diluted 1:10 and 1:20 with adjusted saline likewise gave inconsistent results, although traces of hemoglobin in serum did not interfere noticeably.

Cerebrospinal and Edema Fluids.—The good agreement observed between the two methods confirmed the work of Schaefer and Schmidt (17) on spinal fluid and showed that the quinhydrone electrode was sufficiently stable in poorly buffered material to

TABLE III.

Comparative Results for Various Biological Fluids.

Sample.	Hydrogen electrode.		Quinhydrone electrode.		Difference.
	pH read.	Average.	pH read.	Average.	
Cerebrospinal fluid.	7.44	7.45	7.44	7.45	0.00
	7.45		7.45		
" "	7.40	7.40	7.40	7.41	+0.01
	7.40		7.42		
Edema fluid (unprotected against CO_2 loss).	8.11	8.11	8.08	8.08	−0.03
			8.08		
			8.03		
			8.13		
Urine, sp. gr. 1.022.	5.26	5.26	5.25	5.26	0.00
	5.26		5.25		
			5.29		
" " " 1.003.	6.37	6.37	6.35	6.36	−0.01
			6.37		
" " " 1.015.	5.28	5.28	5.31	5.32	+0.04
			5.32		
" " " 1.022.	5.21	5.21	5.27	5.28	+0.07
			5.28		
" 2 wks. old.	4.86	4.86	4.88	4.91	+0.05
	4.86		4.94		
" strong NH_3 odor.	8.89	8.89	8.40	8.41	−0.48
			8.41		
			8.43		
" NH_4OH added.	6.76	6.76	6.78	6.78	+0.02
			6.78		
"	6.21	6.21	6.27	6.27	+0.06
			6.27		
"	6.74	6.74	6.76	6.77	+0.03
			6.77		
"	8.16	8.16	8.17	8.17	+0.01
			8.17		
Culture medium, adjusted broth.	7.77	7.77	7.76	7.77	0.00
			7.77		
Sterile broth, plain.	5.91	5.91	5.90	5.90	−0.01

TABLE III—*Concluded.*

Sample.	Hydrogen electrode.		Quinhydrone electrode.		Differences.
	pH read.	Average.	pH read.	Average.	
Milk, raw, cow's.	6.33	6.33	6.31	6.31	−0.02
	6.33				
" pasteurized.	6.68	6.68	6.64	6.64	−0.04
	6.68		6.64		
" "	6.64	6.65	6.59	6.60	−0.05
	6.65		6.60		
Cream.	6.72	6.72	6.69	6.70	−0.02
	6.72		6.71		
Milk, pasteurized, 1 day old.	6.90	6.91	6.87	6.87	−0.04
	6.92		6.87		
Milk, pasteurized.	6.63	6.63	6.64	6.64	+0.01
			6.64		
			6.65		
" "	6.68	6.68	6.69	6.69	+0.01
	6.68		6.69		
			6.69		
			6.70		
" "	6.62	6.63	6.63	6.64	+0.01
	6.63		6.64		
" "	6.72	6.72	6.68	6.69	−0.03
	6.72		6.69		
			6.70		
Same, 4 hrs. later.	6.64	6.64	6.64	6.65	+0.01
	6.64		6.65		
Milk, raw.	6.60	6.61	6.59	6.60	−0.01
	6.62		6.60		
Gastric contents, infant, 22 hrs. old.	5.75*	5.80	5.80	5.81	+0.01
	5.84		5.81		
Gastric contents, infant.	4.19	4.19	4.19	4.19	0.00
	4.19				
Same, high mucus content.	5.36	5.36	5.31	5.32	−0.04
			5.32		
Same, marasmus.	5.21	5.22	5.28	5.29	+0.07
	5.23		5.30		

* Hydrogen electrode results for the gastric contents of infants were taken from unpublished studies on gastric secretion by Dr. Joseph Stokes, Jr., and were determined by Miss Julianna Tatum.

make possible the use of the method even when the pH was slightly above 8.0. Drifts were less pronounced than in plasma and serum.

Urine.—The quinhydrone electrode had not previously been compared with the hydrogen electrode for the determination of the hydrion concentration of urine. Table III shows that the two methods agreed well and that the agreement extended for an appreciable distance above pH 8.0. The age or concentration of the urine apparently did not affect the results. A pH of 8.89 was definitely outside of the region in which the quinhydrone electrode gives significant results.

Gastric Contents of Infants.—Hydrion concentration determinations on this material were usually complicated by the smallness of the samples which in addition were frequently of a semisolid nature because of the presence of large amounts of mucus and curd. Colorimetric determinations have been found to be subject to large errors above pH 3.00. However, the quinhydrone electrode compared favorably with the hydrogen electrode even when the viscosity was sufficient to cause the quinhydrone to remain suspended in the material.

Milk.—This proved to be well adapted to quinhydrone electrode determinations, drifts being almost absent. Lester (18) has reached a similar conclusion.

Culture Media.—There appears to be no record of the use of the quinhydrone electrode for culture media. In this material, also, color and turbidity frequently interfere with colorimetric determinations. The results for sterile broth show the method to be well adapted for use with this type of material.

DISCUSSION.

Several differences in regard to material and methods suggest explanations for the differences between the results for human plasma and serum reported in this paper and those of Cullen and Earle. Their results are based chiefly on serum from normal ambulatory subjects, while our data, with one exception, are on plasma or serum from hospitalized subjects. However, several of the latter were completely convalescent and one would think normal so far as the acid-base balance is concerned. It also seems strange that the widely divergent group of pathological conditions represented in these papers should have a common factor which

is capable of affecting the quinhydrone electrode. The fact that serum was used in one group of experiments and plasma in the other is not important since the presence of fibrin has no perceptible effect on the determination. Neither does the use of extrapolation by Cullen and Earle and of direct readings by the writers explain the differences observed, since the results of the former would have been practically the same if the potentials read at 30 seconds had been used for the calculation.

It has been pointed out earlier in this paper that the use of balanced quinhydrone electrodes would presumably eliminate certain errors to which the quinhydrone electrode is subject and reduce others by means of parallel compensating reactions. While the importance of these correcting factors has not been determined, it is possible that they play an important part in eliminating the correction which Cullen and Earle found necessary in order to bring the results of their quinhydrone electrode determinations into agreement with the hydrogen electrode values.

SUMMARY.

It was apparent that the reaction between hemoglobin and quinhydrone prevented the application of the quinhydrone electrode to the determination of the hydrion concentration of whole blood.

For humam serum and plasma the results agreed with those given by the hydrogen electrode within the limits of error of the two methods. A comparison of the two methods in horse serum likewise was satisfactory, but in dog serum deviations from the hydrogen electrode values were of sufficient magnitude to make inadvisable the use of the quinhydrone electrode for accurate results on this material.

Secondary changes in the quinhydrone or its dissociation products hampered somewhat the use of the method for serum and plasma. However, the error from this source could be eliminated by estimating the initial potential which was dependent on the pH. With this precaution the quinhydrone electrode approached the hydrogen electrode in precision and reproducibility.

Good agreement existed between the results of the quinhydrone and the hydrogen electrodes in cerebrospinal fluid, edema fluid, urine, milk, gastric contents of infants, and culture media. The

pH range at which agreement existed between the two methods extended an appreciable distance above pH 8.0 in all of the materials examined.

The balanced titrimetric quinhydrone electrode provides a simple and inexpensive method for hydrion concentration measurements, requiring much less equipment than other quinhydrone methods. It is more accurate than the indicator methods and is sufficiently trustworthy to permit its substitution for the hydrogen electrode for many types of biological work.

The writers wish to express their thanks to Dr. D. Wright Wilson for his helpful criticism and to Dr. W. G. Karr for many suggestions.

BIBLIOGRAPHY.

1. Corran, J. W., and Lewis, W. C. M., *Biochem. J.*, 1924, xviii, 1358.
2. Gesell, R., and Hertzman, A. B., *Am. J. Physiol.*, 1926, lxxviii, 206.
3. Grossman, F., *Biochem. J.*, 1927, xxi, 267.
4. Liu, H. K., *Biochem. Z.*, 1927, clxxxv, 242.
5. Meeker, G. H., and Oser, B. L., *J. Biol. Chem.*, 1926, lxvii, 307.
6. Mislowitzer, E., *Biochem. Z.*, 1925, clix, 77; *Klin. Woch.*, 1926, ii, 1863.
7. Schaefer, R., *Biochem. Z.*, 1926, clxvii, 433.
8. Cullen, G. E., and Biilmann, E., *J. Biol. Chem.*, 1925, lxiv, 727.
9. Greppi, E., and Lucchini, C., *Ber. ges. Physiol. u. exp. Pharmakol.*, 1927, xxxix, 7.
10. Runge, H., and Schmidt, O., *Deutsch. med. Woch.* 1926, lii, 2077.
11. Vellinger, E., and Roche, J., *Bull. Soc. chim. biol.*, 1925, vii, 1004.
12. Cullen, G. E., and Earle, I. P., *J. Biol. Chem.*, 1928, lxxvi, 565.
13. Biilmann, E., and Krarup, I., *J. Chem. Soc.*, 1924, cxxv, 1954.
14. Biilmann, E., and Lund, H., *Ann. Chem.*, 1921, xvi, 321.
15. Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1923.
16. Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.
17. Schaefer, R., and Schmidt, F., *Biochem. Z.*, 1925, clvi, 63.
18. Lester, V., *J. Agric. Sc.*, 1924, xiv, 634.

STUDIES IN THE METABOLISM OF THE BILE.

I. A QUANTITATIVE PETTENKOFER TEST APPLICABLE TO THE DETERMINATION OF BILE ACIDS IN THE BLOOD.*

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The striking progress in our knowledge of the origin and fate of the bile pigments in recent years has been due in great part to the elaboration of methods for studying changes in the bilirubin content of the blood (2, 22, 38, 43). The deficiencies in our knowledge of the physiology of the bile acids by contrast are even more striking. Progress in this field of physiology and medicine may be facilitated by a satisfactory method for determining bile acids, particularly in small amounts of blood.

Methods have been devised for determining glycocholic and taurocholic acids in the bile or urine, and Foster and Hooper (7), Symth and Whipple (39), Brakefield and Schmidt (5), and Rosenthal and his associates (32–36) have studied the effect of various physiologic influences and experimental conditions on the excretion of these compounds in the bile or urine. Unfortunately, the procedures used by these authors are not sufficiently sensitive to be readily applicable to blood.

Kühne (17) and Huppert (15) reported the isolation of bile acids from the blood following both biliary obstruction and their intravenous injection. Various other experimenters have obtained qualitative color tests for bile acids in the blood. Moleschott (26), Lehmann (18), Blankenhorn (4), Gilbert, Chabrol, and Bénard (8), and Petrén (30), and others (14, 16) found

* The data presented in this paper dealing with the development of the Pettenkofer reaction are taken from a thesis submitted by Miss Aldrich to the faculty of the Graduate School of the University of Minnesota in partial fulfilment of the requirements for the degree of Master of Science. Reported in part before the American Physiological Society, Rochester, New York, April, 1927 (9, 10). The comparison with the accepted methods for the analysis of bile has been made by Miss Bledsoe.

positive Pettenkofer tests under appropriate conditions. Recently Tashiro (41), and Herzfeld and Haemmerli (12, 13) reported partially successful attempts to adapt this test to quantitative determinations. Perlzweig and Barron (29) reported a new colorimetric test for bile acids using acetic anhydride and sulfuric acid; Szilard (40) precipitates the bile acids with ferric chloride and determines the iron colorimetrically; Rosenthal and Wislicki (36) use a modification of the gasometric method for the determination of amino acids in the bile; McNee (25) has suggested a similar method.

Because of the difficulties in the application of chemical tests, Adler (1) and others turned to physical methods and have reported extensive studies on the surface tension of serum. A review of these methods, however, showed that they were unsuitable for our proposed study because they either were not sensitive or specific enough or were not applicable clinically.

The Pettenkofer (31) test is probably the most sensitive reaction known at present for the bile acids. As such it offers the best basis for a method which can be readily adapted for use with small amounts of blood. One of us (Aldrich) has been able to develop a quantitative modification of the Pettenkofer test suitable for such use.[1] The quantities of bile acids found in the blood even under experimental conditions are too small to permit a satisfactory comparison of this method with those previously used for the analysis of bile. The modified Pettenkofer reaction is equally applicable to bile and a comparison with these methods is reported later.

Quantitative Adaptation of the Pettenkofer Test.

The method here described is a quantitative adaptation by which the maximal Pettenkofer value obtainable from the blood is determined. Since the technique is comparatively simple and only 5 cc. of blood are used, it is capable of direct clinical application. We have carried out more than 2000 determinations on the blood in various clinical and experimental conditions during the last 2 years.

5 cc. of oxalated whole blood are added with shaking to 35 cc. of redistilled 95 per cent alcohol in a 50 cc. volumetric flask. The contents are made to volume with alcohol and filtered. From 40 to 50 mg. of norit are added to 35 cc. of the slightly colored filtrate,

[1] This standardization of the Pettenkofer reaction was undertaken at the suggestion of Dr. L. G. Rowntree and carried out under the supervision of Dr. C. H. Greene.

shaken well, and filtered at once. The resulting solution should be clear and colorless.

30 cc. of this filtrate are transferred to a 100 cc. beaker, 1 cc. of a saturated solution of barium hydroxide is added, and the mixture evaporated rapidly to a volume of 3 or 4 cc. on a hot electric plate. Overheating must be avoided and the final drying is best accomplished with an electric fan. To remove cholesterol and fat, the thoroughly dried residue of barium salts is then extracted three times with 5 cc. portions of redistilled anhydrous ether brought to the boiling point on the hot plate each time. Fragments of the residue loosened during this extraction are recovered by centrifuging the ether washings. Recrystallized glycocholic acid is used as a standard. A 0.1 per cent alcoholic stock solution is diluted 10 times with alcohol, so that the resulting standard solution contains 0.1 mg. of glycocholic acid for each cc. Five standards containing 0.1, 0.15, 0.2, 0.3, and 0.4 mg., respectively, are prepared, and the solutions evaporated to dryness by placing the beakers before an electric fan.

To the dried unknown and to each standard is added 0.4 cc. of a 1 per cent solution of cane sugar which, in the unknown, should be rubbed up with the barium precipitate. 8 cc. of 60 per cent (by volume) sulfuric acid are added to each beaker and the contents mixed. The loosened precipitate recovered from the ether washings is washed from the centrifuge tube to the respective beaker at this time. The beakers are then placed in an oven or water bath at approximately 37° for 1 hour. It is during this period that the solution becomes pink. The solutions are then cooled in the ice box for a few minutes to retard further development of color. During the development of the Pettenkofer color, and thereafter, the unknown and standard solutions must be treated alike with respect to light and heat. The barium sulfate precipitate in the unknown is removed by centrifuging at high speed for 15 minutes. The supernatant solution is then read in a colorimeter against the standard most nearly corresponding in intensity.

The color developed in the Pettenkofer reaction varies from a faint pinkish yellow to a deep purplish pink as the amount of bile acids in the solution increases. Because of this change in hue, direct colorimetric comparison is difficult. Satisfactory results have been obtained with a Duboscq colorimeter, fused glass cups

and a compensating wedge being used. The standard which most nearly corresponds in intensity to the unknown is set at 15 mm. in the left hand cup, and the unknown matched with it. The compensating wedge is used to secure an exact correspondence in hue of the two solutions.

The strength of the Pettenkofer reaction is reported in terms of mg. of glycocholic acid for each 100 cc. of blood calculated by the formula: $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times$ strength of standard in mg. $\times \frac{100}{3}$ = mg. of glycocholic acid for each 100 cc. of blood.

Preparation of Crystalline Glycocholic Acid.[2]

Commercial preparations of glycocholic acid sufficiently pure for use as a standard have been difficult to obtain and we have found it necessary to make our own preparations. For this purpose, a 5 per cent solution of a commercial preparation of the bile salts obtained from Armour and Company was used. The glycocholic acid in this solution was then precipitated by the addition of an excess of a 10 per cent ferric chloride solution according to the directions of Hammarsten (11). The curdy precipitate was filtered off in a Buchner funnel and washed once with water. The iron salt was then decomposed with 5 per cent sodium carbonate solution. Care must be taken to rub up the precipitate thoroughly with several changes of the carbonate solution; otherwise there will be considerable loss at this stage. The solution containing the sodium glycocholate was then neutralized to litmus with hydrochloric acid and evaporated on a steam bath to a small volume. Ether was poured on the solution to a depth of 1 to 2 cm., and dilute hydrochloric acid added slowly, with vigorous shaking, until permanent opalescence was produced. It was then placed on ice until crystallization started. Once this was started more acid was added and the solution almost immediately became transformed into a firm mass of fine needle-like crystals. The mother liquor was removed by a suction filter. The crystals were redissolved with the aid of sodium carbonate to form approximately a 10 per cent solution and recrystallized by the addition of ether

[2] This has been prepared by Dr. C. H. Greene.

and acid. In crystallization an excess of acid is to be avoided, and the process is greatly facilitated by "seeding" with crystals of glycocholic acid. The first crop of crystals was usually white; if not, the crystals were dissolved in alcohol and decolorized by charcoal. The material was recrystallized three times as outlined. It was then recrystallized twice from absolute alcohol by the addition of distilled water. The final product was of acceptable purity as judged by the analytic figures given in Table I. Letsche (19) found that the melting point is not sharp and depends on the rate of heating. The values reported are for the temperature at which the material sinters. He has pointed out that care must be used in the final drying of the crystals to avoid the formation of paraglycocholic acid, which is present in all specimens which have

TABLE I.

Analysis of Preparations of Crystalline Glycocholic Acid.

	Total N.	Amino N.	Melting point.*	$[\alpha]_D^{20}$ in alcohol.	S	P
	per cent	*per cent*	*°C.*	*degrees*	*per cent*	*per cent*
Theory for $C_{26}H_{43}O_6N$.....	3.01	3.01	125–127	+32.3	0.00	0.00
Preparation 1 (used as standard).....	2.96	2.97	125–128	+32.3	0.00	0.027
Preparation 2.....	3.14	2.68	126–150	+31.7	1.10	0.032

*Uncorrected.

stood for some time. The formation of the latter markedly elevates the melting point but it gives the same reaction with the Pettenkofer test as glycocholic acid and its presence in a sample of glycocholic acid does not interfere with the use of the latter as a standard.

Phosphatides are reported to give a positive Pettenkofer reaction, and Long and Gephart (21) have pointed out the difficulty in eliminating all traces of them from preparations of the bile acids. The recrystallized glycocholic acid used by us as a standard contained only 0.027 per cent of total phosphorus. This is equivalent to less than 1 per cent of lecithin, a negligible amount so far as this method is concerned.

Control Experiments on Method.

Development of Color.—The accuracy of any colorimetric method depends on the uniformity of the color and on the correctness of the proportionality between the color and the amount of material to be determined. In the Pettenkofer test the sulfuric acid and sugar form a furfural derivative, which, under the influence of the sulfuric acid, reacts with cholic acid to produce a purplish pink color. The development of the color is a progressive reaction, the speed of which can be controlled by temperature. For the quantitative test this is most satisfactorily controlled by first evaporating the solution to be tested to dryness; sugar in a small volume of solution (4 mg. in 0.4 cc.) and 60 per cent sulfuric acid are then added to the residue at room temperature and the temperatures of all the unknown and standard solutions are raised simultaneously.

Different degrees of color are developed by different degrees of heat but, whatever the condition of heat, it must be exactly the same for all tests, in order to secure proportionality in the intensity of the color. Variations in the temperature of different sections of a drying oven or incubator may lead to considerable variation in the color developed. The optimal conditions of heating were secured in a water bath of uniform temperature. To standardize the heating more accurately the same size and shape of receptacle should be used in all tests. 100 cc. beakers are convenient for the volumes recommended.

With the same amount of bile acids the hue and intensity of color are dependent not only on the temperature but on the concentration of the reagents. Sugar was found to be a more satisfactory reagent than furfural, which has been used by certain investigators, since furfural did not increase the sensitivity of the test and is more difficult to standardize. 4 mg. of sugar are suitable for routine use, since the color is good and the intensity adequate for colorimetry. The solution containing 60 per cent sulfuric acid was found to be the most satisfactory for colorimetry in regard to both the hue and intensity of the color produced.

The uniformity of the color developed by this technique and the proportionality in intensity between the color and the amount of bile acids used is shown by the comparison of various standard

solutions in Table II. This table, which gives a typical series of results, shows variations in most cases not exceeding 3 per cent. These are due in part to variations in the colors developed because of unequal heating and in a slight degree to the difficulties of the colorimetric comparison of solutions of varying hue. Slight changes in the concentration of bile acids produce marked changes in the hue. It is essential, therefore, that the standard and unknown be of approximately the same concentration, but it is manifestly impossible to prepare standards which will match all concentrations of bile acids. Furthermore, the blood tests are slightly yellower than the corresponding standard solutions.

TABLE II.

Uniformity and Proportionality of Color Developed by Pettenkofer Reaction Under Standardized Conditions.

Observed Colorimetric Readings of Standard Solutions of Varying Concentrations.

0.1 mg. glycocholic acid.		0.15 mg. glycocholic acid.		0.20 mg. glycocholic acid.		0.25 mg glycocholic acid.		0.30 mg. glycocholic acid.	
Against 0.1 mg. at 15 mm. (theory, 15.0).	Against 0.15 mg. at 15 mm. (theory, 22.5).	Against 0.15 mg. at 15 mm. (theory, 15.0).	Against 0.20 mg. at 15 mm. (theory, 20.0).	Against 0.20 mg. at 15 mm. (theory, 15.0).	Against 0.25 mg. at 15 mm. (theory, 18.75).	Against 0.25 mg. at 15 mm. (theory, 15.0).	Against 0.30 mg. at 15 mm. (theory, 18.0).	Against 0.30 mg. at 15 mm. (theory, 15.0).	Against 0.40 mg. at 15 mm. (theory, 20.0).
15.5	23.5	15.0	20.0	15.0	18.7	15.0	17.8	15.0	19.8
15.0	23.4	15.5	20.4	15.0	18.7	15.0	17.5	15.3	20.0
14.8	22.2	15.0	20.0	15.0	18.3	15.0	18.0	14.7	20.2
14.7	22.5	15.0	20.0	14.7	18.7	14.8	18.2	15.3	20.5

Especial attention, therefore, must be given to the colorimetric readings, which should be made within an hour after the development of the color. On standing at room temperature, the solutions slowly become browner, the rapidity of the change depending on the temperature.

Colorimetric Comparison.—For an accurate comparison of intensity of colors by colorimetry the hue and transparency of the solutions to be compared should correspond exactly. In the case of the Pettenkofer reaction it is essential that full compensation for the yellow be made if the intensity of the pink is to be determined quantitatively. This is especially necessary in blood tests

in which unavoidably the tint is yellower than the corresponding standards. Satisfactory readings were obtained with a Duboscq colorimeter by means of a compensating wedge of the type used in the Autenrieth-Hellige colorimeter. The wedge was filled with a solution prepared by heating 0.4 cc. of the sugar solution and 8 cc. of 60 per cent sulfuric acid for 1 hour at 37°. A second wedge was filled with this solution diluted from one-third to one-half with water. These colors are quite stable and can be used over a long period of time.

Because of the strong sulfuric acid a colorimeter equipped with fused glass cups is essential. The unknown solution is then read against the standard which most nearly corresponds in color. The yellow wedge is held beneath the pinker of the two solutions (usually the standard) and so regulated as to secure an exact equivalence in hue. The colorimetric reading is laborious but after some practice a fair degree of speed and accuracy can be obtained. Satisfactory readings can also be obtained by the use of a bicolorimeter such as that described by Myers (27) or by Wu (44).

Comparison of the Pettenkofer Test with Previously Accepted Methods for Determination of Bile Acids in Bile.—The Pettenkofer reaction provides a most sensitive test for the bile acids but because this reaction is not entirely specific its use for the determination of these substances in biologic fluids has been criticized. While the method reported here was developed primarily for the analysis of blood it is equally applicable to bile (23, 24). As such it is possible to control the results obtained by comparison with the accepted methods for the determination of the bile acids in bile.

Samples of bile were extracted with boiling alcohol, cooled, and made up to a volume corresponding to a 1:200 dilution. The filtered solution was then decolorized by adding norit in the proportion of 50 mg. for each 35 cc. An aliquot supplying 0.2 to 0.4 mg. of bile acids was then evaporated to dryness and treated with acid as were the blood samples. The norit removes nearly all the cholesterol from the bile sample and in consequence treatment by barium hydroxide and subsequent extraction with ether are unnecessary.

For comparison with the Pettenkofer reaction in the latter case

we have used the method of Schmidt and Dart (37), a modification of the original method of Foster and Hooper (7), in which the bile acids are hydrolyzed by alkali and the liberated taurine and glycine determined by the gasometric amino acid method of Van Slyke. The method of Rosenthal and Lauterbach (35) is similar except that the β-naphthoquinone-sulfonic acid reagent of Folin (6) is used for the colorimetric determination of the liberated amino acids. In each case we have determined the neutral sulfur content of the protein-free bile and calculated the taurocholic acid present by the method of von Bergmann (3). By making appropriate corrections in each case, it is then possible to calculate the glycocholic acid present.

The comparison of the three methods as applied to the analysis of commercial preparations of bile salts and specimens of bile from different sources is shown in Table III. In each group, one set of analyses in triplicate is given to show the variations of each method. To simplify the presentation of this material only the averages of such determinations are given in the remainder of the table. The composition of the different specimens of bile agrees with the usually accepted values while the extreme variability in the composition of commercial preparations of bile salts, a point emphasized by Lewis (20), is well illustrated.

The gasometric method for the determination of amino nitrogen can be used on colored solutions such as the alcoholic extract of bile, but the pigment must be removed if the amino nitrogen liberated by hydrolysis is to be determined colorimetrically or if the Pettenkofer test is to be used for analysis. Charcoal has been used commonly to decolorize the bile. Table III shows that charcoal, when used in excess, may cause a loss of from 2 to 10 per cent in the amino nitrogen liberated by hydrolysis in bile samples. The sulfur content of dog bile can be used as an index to the taurocholic acid present, and sulfur determinations show a corresponding loss when the bile is treated with charcoal. An excess of charcoal, therefore, may be a source of loss, but if minimal amounts are used, this does not introduce an error greater than is inherent in the method, and the analytic results obtained by the use of the modified Pettenkofer test generally agree with those obtained by the gasometric method which does not require the use of charcoal.

TABLE III.

Comparative Analyses of Bile and Preparations of Bile Salts.

Glycocholic acid.	S	Taurine N calculated from average for S.	Gasometric determination of amino N (Schmidt and Dart), per cent.							Colorimetric determination of amino N (Rosenthal and Lauterbach), per cent.						Quantitative Pettenkofer test (Aldrich), per cent.				
			Total amino N.	Calculated taurine N.	Glycine N, uncorrected.	Glycine N, corrected.	Taurocholic acid calculated from S.	Glycocholic acid.	Total bile acids.	Total amino N.	Calculated taurine N.	Glycine N.	Taurocholic acid calculated from S.	Glycocholic acid.	Total bile acids.	Total bile acids calculated as glycocholic acid.	Taurocholic acid calculated as glycocholic acid.	Taurocholic acid calculated from S.	Glycocholic acid.	Total bile acids.
	per cent																			
Preparation 1 (standard).....	0.00	0.00	2.98	0.00	2.98	2.90	0.0	96.0	96.0	2.67	0.00	2.67	0.0	89.0	89.0	100.0		0.0	100.0	100.0
Preparation 2.....	1.10	0.47	2.76	0.47	2.29	2.22	17.3	73.4	90.7	2.47	0.32	2.15	17.3	71.6	88.9	90.7	15.6	17.3	75.1	92.4
Commercial bile salt preparations.																				
1	3.25	1.40	1.95	1.40	0.55	0.53	51.8	17.6	69.4	1.17	0.93	0.24	51.8	8.0	59.8	66.0	47.7	51.8	18.3	70.1
	3.21		1.88		0.48	0.47		15.6	67.4	1.18		0.25		8.3	60.1	65.0			17.3	69.1
	3.24		1.90		0.50	0.48		16.0	67.8	1.19		0.26		8.6	60.4	64.5			16.8	68.6
2	3.57	1.55	2.08	1.55	0.53	0.51	57.2	17.1	74.3	1.72	1.03	0.69	57.2	26.9	84.1	74.1	51.6	57.2	22.5	79.7
3	2.45	1.07	1.96	1.07	0.89	0.87	39.3	28.8	68.1	1.25	0.71	0.54	39.3	18.1	57.4	64.6	35.4	39.3	29.2	68.5
4	0.48	0.21	1.09	0.21	0.88	0.85	7.7	28.2	35.9	0.80	0.14	0.66	7.7	21.9	29.6	31.3	6.9	7.7	24.4	32.1

3	2.45	1.07	1.96	1.07	0.89	0.87	39.3	28.8	68.1	1.25	0.71	0.91								
4	0.48	0.21	1.09	0.21	0.88	0.85	7.7	28.2	35.9	0.80	0.14	0.66	7.7	21.9	29.6	31.3	6.9	7.7	24.4	32.1

Bile samples (from hospital patients).																				
1. Gallbladder....	0.106	0.047	0.171	0.047	0.124	0.120	1.72	3.98	5.70	0.113	0.032	0.081	1.72	2.69	4.41	5.17	1.55	1.72	3.62	5.34
	0.113		0.174		0.127	0.123		4.08	5.80	0.113		0.081		2.69	4.41	5.25			3.70	5.42
	0.102		0.172		0.125	0.121		4.02	5.74	0.119		0.087		2.90	4.62	5.08			3.53	5.25
2. Bile fistula.....	0.065	0.028	0.087	0.028	0.059	0.057	1.04	1.90	2.94	0.065	0.018	0.047	1.04	1.56	2.60	2.74	0.94	1.04	1.80	2.84
3. " "	0.043	0.018	0.057	0.018	0.038	0.038	0.67	1.26	1.93	0.045	0.012	0.033	0.67	1.10	1.77	1.90	0.60	0.67	1.30	1.97
4 a. Gallbladder untreated.....	0.098	0.043	0.125	0.043	0.082	0.079	1.57	2.62	4.19	0.113	0.029	0.084	1.57	2.80	4.37	3.88	1.41	1.57	2.47	4.04
4 b. After treatment with charcoal.....	0.90	0.039	0.123	0.039	0.084	0.081	1.45	2.65	4.10	0.102	0.026	0.076	1.45	2.52	3.97	3.70	1.31	1.45	2.39	3.84
Bile samples (from dogs).																				
1. Gallbladder.	0.90	0.40	0.35	0.40			14.8	0.0	12.9	0.26	0.27		14.8	0.0	14.4	13.1	13.4	14.8		14.5
	0.94		0.35						12.9	0.25					13.8	14.2			0.8	15.6
	0.91		0.35						12.9											
2. Gallbladder....	0.85	0.37	0.33	0.37			13.6	0.0	12.2	0.15	0.24		13.6	0.0	7.8	13.4	12.3	13.6	1.1	14.7
3. Bile fistula.....	0.101	0.043	0.039	0.043			1.61	0.0	1.44	0.026	0.029		1.61	0.0	1.44	1.45	1.45	1.61		1.61
4 a. Bile fistula untreated.....	0.336	0.146	0.144	0.146			5.40	0.0	5.31	0.053	0.097		5.40	0.0	2.92	4.92	4.87	5.40	0.05	5.45
4 b. After treatment with charcoal.....	0.329	0.143	0.132	0.143			5.28	0.0	4.86	0.047	0.095		5.28	0.0	2.60	4.73	4.76	5.28		5.25

Rosenthal and Lauterbach (35) report that the reaction of taurine with the Folin amino acid reagent produces only two-thirds of the expected amount of color. We have found this reaction to be much more irregular. The color is yellower than that obtained from alanine or glycine standards, and the intensity also seems to vary with the quantity of taurine present and its relationship to the total amino acids. We used the same correction factor in calculating the taurine color as did Rosenthal and Lauterbach but believe that it is but an approximation at best. The analysis of dog bile in particular emphasizes the disparity between the taurocholic acid so determined and that calculated from the sulfur present.

In general these three methods give concordant results, especially in the analysis of bile obtained from man. The method of Rosenthal and Lauterbach is more variable than the other two and is not reliable in the analysis of dog bile. The difference between the results obtained with the modified Pettenkofer test and the Foster and Hooper (7) method as modified by Schmidt and Dart (37) are within the limits of error of either. As such they would seem to be equally useful for the proximate analysis of bile. The assumed lack of specificity of the Pettenkofer test will always cause adverse criticism, yet comparison of the results obtained by the Pettenkofer method and that of Foster and Hooper indicates that, in the alcoholic extract of bile, extraneous substances do not interfere to an appreciable extent with the determinations of bile acids by this modification of the Pettenkofer test.

Application of Method to Blood.

Extraction of Bile Acids from Blood.—The bile acids occur in the blood as alkaline salts which are readily soluble in water or alcohol. Redistilled alcohol was used in the method to precipitate the blood proteins and extract the bile salts. Similar values were obtained both with hot and cold alcohol extraction; heating is therefore unnecessary.

The Pettenkofer reaction is not a specific test for bile acids. Von Udranszky (42), Mylius (28), and others, have pointed out numerous other compounds which give a similar color, among them lecithin, cholesterol, and oleic acid which may be present in an alcoholic extract of blood. As shown later, the Pettenkofer

reaction of these substances under the conditions of our test is minimal, but we have not been able to extract bile acids from the blood quantitatively without having to include some of these substances in the extract. The method, therefore, has been developed to give the maximal Pettenkofer test in the protein-free blood filtrate.

The alcoholic filtrate may be colored even when no bile pigment is present and this coloring matter interferes with the subsequent Pettenkofer test. No solvent was found that would extract bile acids without likewise extracting considerable quantities of pigment. Various methods of decolorizing the alcoholic filtrate were tried; the results indicate that the activated carbon, norit, could be used more satisfactorily than the others tested.

The optimal amount of norit seems to be the smallest quantity that will free the filtrate of interfering pigment. Norit will remove bile acids from pure solutions and, as we have pointed out, its use in excess in decolorizing bile causes a slight loss. However, charcoal is less active in removing bile salts from blood filtrates containing pigments and other readily adsorbable substances. On an average more than 90 per cent of bile acids added to blood filtrates can be recovered after clearing with 50 mg. of norit and for routine use this amount of norit added to 35 cc. of blood filtrate is optimal.

The clear alcoholic filtrate, evaporated to dryness, treated with sugar and sulfuric acid, and heated, causes the pink color characteristic of the Pettenkofer reaction. With this technique some lecithin, cholesterol, and fat are to be found in the solution. These substances have been reported as giving a positive Pettenkofer test; they were, therefore, studied.

Fatty acids and cholesterol, in amounts equivalent to those present in the extract of a blood sample containing 400 mg. of the former and from 200 to 900 mg. of the latter for each 100 cc., respectively, when evaporated to dryness and treated with sugar and sulfuric acid under the conditions of this test did not give a positive test. Samples of commercial lecithin gave a slight color, the purest sample producing about one three-hundredths of the color which would be developed by the same weight of bile acids. The only other substance which gave a positive Pettenkofer test was a sample of cholesterol which had been in the laboratory for

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several years. The introduction of a Pettenkofer color developed by such substances apparently is not a serious source of error. They do, however, add to the organic substances which, when charred by the sulfuric acid, produce an interfering yellow tint in

TABLE IV.

Recovery of Bile Acids Added to Whole Blood.

Sample No.	Glycocholic acid, mg. per cent.					Difference.
	Initial blood sample.	Acid added.	Total theoretic values.	Observed values.	Difference between observed and theoretic values.	
						per cent
1	4.0	4.0	8.0	7.9	−0.1	−1.2
				7.8	−0.2	−2.5
2	4.2	4.0	8.2	7.7	−0.5	−6.1
3	4.0	4.0	8.0	7.9	−0.1	−1.2
				8.0	±0.0	±0.0
4	4.7	4.0	8.7	8.0	−0.7	−8.0
				8.8	+0.1	+1.1
5	4.7	4.0	8.7	8.6	−0.1	−1.1
6	4.0	6.0	10.0	8.9	−1.1	−11.0
				9.1	−0.9	−9.0
7	4.2	6.0	10.2	9.8	−0.4	−3.9
8	4.0	6.0	10.0	8.9	−1.1	−11.0
9	4.7	6.0	10.7	10.0	−0.7	−6.5
				10.7	±0.0	±0.0
10	4.7	6.0	10.7	9.7	−1.0	−9.4
11	4.0	8.0	12.0	11.1	−0.9	−7.5
12	4.2	8.0	12.2	12.2	±0.0	±0.0
13	4.0	8.0	12.0	10.4	−1.6	−13.3
				11.7	−0.3	−2.5
14	4.7	8.0	12.7	12.1	−0.6	−4.7
				12.6	−0.1	−7.8
15	5.0	8.0	13.0	12.4	−0.6	−4.6
				12.4	−0.6	−4.6
16	3.4	8.0	11.4	11.4	±0.0	±0.0

the test solution. This makes the colorimetric reading difficult, particularly in blood giving only a faint Pettenkofer test. Their removal was therefore attempted by ether extraction.

Although pure bile salts are relatively insoluble in ether,

amounts equivalent to those studied here are readily soluble in ether containing fats or other lipoids. The addition of a small amount of barium hydroxide does not prevent this loss. Petrén cleared an alcoholic blood filtrate with dry powdered barium hydroxide and evaporated it to dryness. After extracting with ether to remove the fats he found that the ether extract gave a positive Pettenkofer reaction, and so questioned the use of this test. These experiments were repeated and confirmed by us. Furthermore we found that bile acids added to the alcoholic filtrate prepared according to Petrén's method are largely removed when the dried residue is extracted with ether. The addition of 1 cc. of a saturated solution of barium hydroxide to the blood filtrate prevented any such loss of bile acids during the extraction with ether. Analysis showed this ether extract to contain cholesterol and some fats but no lecithin. No further separation of the bile acids from the organic substances that escaped ether extraction was attempted. Even with this degree of separation the color was improved in quality and higher Pettenkofer values were obtained. The addition of baryta and the washing with ether had no effect on the Pettenkofer reaction of the standard glycocholic acid.

Recovery of Bile Acids Added to Blood.—The accuracy of the method as a whole is shown in Table IV, which gives the results obtained in the recovery of known quantities of bile acids added to blood. The average loss was about 0.5 mg. for each 100 cc.

Comment.

We fully realize the difficulties which arise in interpreting data obtained by means of a non-specific color reaction such as the Pettenkofer test for bile acids. Although, so far as possible, interfering substances have been removed, we cannot attach a precise chemical identity to all the material responsible for the Pettenkofer reaction, especially in normal blood. Only the isolation and chemical identification of pure bile acids from normal blood will conclusively prove their presence. By the technique described, the maximal reading in the alcoholic extract of the blood is obtained. In man the blood normally gives a positive Pettenkofer reaction equivalent in intensity to that which would be

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produced by the presence of from 3 to 6 mg. of glycocholic acid for each 100 cc. Increased values have been found in abnormal blood. Table V shows the values obtained in a variety of clinical and experimental conditions.

In conditions in which it is definitely accepted that bile acids are present in the blood in increased amounts, as following their intravenous injection, or in obstructive jaundice, the method is of value in the quantitative study of the changes which occur. Since bile acids added to the blood may be recovered with an accuracy of 90 per cent or more, a normal Pettenkofer value assuredly excludes any significant increase in the blood. Keeping

TABLE V.

Pettenkofer Values of Blood Obtained in a Variety of Conditions.

Condition.	Glycocholic acid.
	mg. per cent
Normal blood (dog)	6.5
Experimental obstructive jaundice in dog	16.3
Following intravenous injection of bile acids in dog	75.0
Normal blood (man)	5.1
" " "	3.2
Obstructive jaundice	8.3
" "	16.7
Carcinoma of liver	15.0
Portal cirrhosis	7.2
Biliary "	14.3

in mind the limits to the interpretation of the values, one may use this modification of the Pettenkofer test to obtain valuable information on the metabolism of the bile acids by the quantitative study of the changes in the blood in various experimental and clinical conditions.

SUMMARY.

The Pettenkofer reaction can be used to determine pure bile acids quantitatively in amounts of from 0.10 to 0.50 mg. with an accuracy of ±5 per cent.

A series of analyses of commercial preparations of bile salts and of bile from different sources, made to compare this method with

that of Schmidt and Dart, shows that the results obtained by the modified Pettenkofer test agree within the limits of error with those obtained by the gasometric determination of the amino nitrogen liberated by alkaline hydrolysis. Because of the rapidity and ease of the determination by the new colorimetric method and because it can be applied in the analysis of small amounts of material, it is the method of choice when these factors enter into the selection of a method.

The reaction was applied to an alcoholic extract of 5 cc. of blood and a method of extraction which permits the determination of maximal amounts of Pettenkofer-reacting material is described.

Recovery of bile acids added to the blood is made by this method with an average loss of about 0.5 mg. for each 100 cc. With few exceptions the recovery is greater than 90 per cent.

When interfering substances are removed as completely as possible, normal blood yields a Pettenkofer value equivalent to from 3 to 6 mg. of glycocholic acid for each 100 cc. of blood. Increased values have been found under certain clinical and experimental conditions.

BIBLIOGRAPHY.

1. Adler, A., Über Verhalten und Wirkung von Gallensäuren in Organismus, *Z. ges. exp. Med.*, 1925, xlvi, 371.
2. van den Bergh, A. A. H., Der Gallenfarbstoff im Blute, Leiden, 1918, viii, 111.
3. von Bergmann, G., Die Überführung von Cystin in Taurin im tierischen Organismus, *Beitr. chem. Physiol. u. Path.*, 1903, iv, 192.
4. Blankenhorn, M. A., The distribution of bile in certain types of jaundice, *Arch. Int. Med.*, 1918, xxi, 282.
5. Brakefield, J. L., and Schmidt, C. L. A., Studies on the synthesis and elimination of certain bile components in obstructive jaundice, *J. Biol. Chem.*, 1926, lxvii, 523.
6. Folin, O., A system of blood analysis. Supplement III. A new colorimetric method for the determination of the amino-acid nitrogen in blood, *J. Biol. Chem.*, 1922, li, 377.
7. Foster, M. G., and Hooper, C. W., The metabolism of bile acids. I. A quantitative method for analysis of bile acids in dog's bile, *J. Biol. Chem.*, 1919, xxxviii, 355.
8. Gilbert, C., Chabrol, E., and Bénard, H., La cholémie saline dans les ictères, *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1602.
9. Greene, C. H., and Aldrich, M., Studies in the metabolism of the bile acids, *Am. J. Physiol.*, 1927, lxxxi, 480.

XUM

10. Greene, C. H., Aldrich, M., and Rowntree, L. G., Studies in the metabolism of the bile acids, *J. Physiol.*, 1927, lxiv, 7.
11. Hammarsten, O., Darstellung der Gallensäuren und ihrer wichtigsten Abbauprodukte und ihr Nachweis, in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, VI. Abt., Berlin and Vienna, 1925, pt. 1, 211.
12. Herzfeld, E., and Haemmerli, A., Die Galle im Stoffwechsel. Eine neue quantitative Gallensäuren-Bestimmungsmethode, *Schweiz. med. Woch.*, 1924, liv, 141.
13. Herzfeld, E., and Haemmerli, A., Die Galle im Stoffwechsel, *Schweiz. med. Woch.*, 1925, lv, 142, 164.
14. Hoover, C. F., and Blankenhorn, M. A., Dissociated jaundice, *Arch. Int. Med.*, 1916, xviii, 289.
15. Huppert, H., Ueber das Schicksal des Gallensäuren im Icterus, *Arch. d. Heilk.*, 1864, v, 236.
16. Kufferath, Ueber die Abwesenheit der Gallensäuren im Blute nach dem Verschluss des Gallen- und des Milchbrustganges, *Arch. Anat. u. Physiol., Physiol. Abt.*, 1880, 92.
17. Kühne, W., Beiträge zur Lehre vom Icterus, *Virchows Arch. path. Anat.*, 1858, xiv, 310.
18. Lehmann, C. C., Lehrbuch der physiologischen Chemie, Leipsic, 2nd edition, 1850, i, 232.
19. Letsche, E., Glykocholsäure und Paraglykocholsäure, *Z. physiol. Chem.*, 1911, lxxiii, 308.
20. Lewis, W. C. M., On variations observed in the composition of sodium glycocholate prepared by different methods, *Biochem. J.*, 1908, iii, 119.
21. Long, J. H., and Gephart, F., On the behavior of lecithin with bile salts, and the occurrence of lecithin in bile, *J. Am. Chem. Soc.*, 1908, xxx, 1312.
22. Mann, F. C., Sheard, C., and Bollman, J. L., Studies on the physiology of the liver. I. The extrahepatic formation of bilirubin, *Am. J. Physiol.*, 1925, lxxiv, 49.
23. McClure, C. W., Huntsinger, M. E., and Montague, O. C., Studies in liver function. III. Methods for determining the furfurol number and the bilirubin concentration of duodenal contents, *Boston Med. and Surg. J.*, 1925, cxciii, 1050.
24. McClure, C. W., Vance, E., and Greene, M. C., Studies in liver function. I. Methods for determining the concentration of bile acids and of pigments present in duodenal contents, *Boston Med. and Surg. J.*, 1925, cxcii, 431.
25. McNee, J. W., personal communication.
26. Moleschott, J., Bilin im Blute, *Z. rational Med.*, 1847, vi, 387.
27. Myers, V. C., A colorimeter for bicolorimetric work, *J. Biol. Chem.*, 1922, liv, 675.
28. Mylius, F., Zur Kenntniss der Pettenkofer'schen Gallensäurereaction, *Z. physiol. Chem.*, 1887, xi, 492.

29. Perlzweig, W. A., and Barron, E. C., New colorimetric method for determination of bile acids in blood, *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 233.
30. Petrén, G., Untersuchungen über die Blutgerrinnung bei Icterus, *Beitr. klin. Chir.*, 1920, cxx, 501.
31. Pettenkofer, M. J., Notiz ueber eine neue Reaction auf Galle und Zucker, *Ann. Chem. u. Pharm.*, 1844, lii, 90.
32. Rosenthal, F., and von Falkenhausen, M. F., Über eine quantitative Bestimmung der Glykocholsäure und Taurocholsäure in der menschlichen Duodenalgalle, *Klin. Woch.*, 1923, ii, 1111.
33. Rosenthal, F., and von Falkenhausen, M. F., Beiträge zur Physiologie und Pathologie der Gallensekretion. I. Über eine quantitative Bestimmung der Gallensäuren in der menschlichen Duodenalgalle, *Arch. exp. Path. u. Pharmakol.*, 1923, xcviii, 321.
34. Rosenthal, F., von Falkenhausen, M. F., and Freund, H., Weitere Beiträge zur Physiologie und Pathologie des Gallensäurensekretion beim Menschen. V. Über das Phänomen des Umkehr der Gallensäurenrelationen in der Galle von Leberkranken, *Arch. exp. Path. u. Pharmakol.*, 1925–26, cxi, 170.
35. Rosenthal, F., and Lauterbach, F., Beiträge zur Physiologie und Pathologie der Gallensekretion. IV. Über eine quantitative kolorimetrische Bestimmung der Gallensäuren in menschlichen Körperflüssigkeiten, *Arch. exp. Path. u. Pharmakol.*, 1924, ci, 1.
36. Rosenthal, F., and Wislicki, L., Über eine quantitative Bestimmung der Gallensäuren im Blute, *Arch. exp. Path. u. Pharmakol.*, 1926, cxvii, 8.
37. Schmidt, C. L. A., and Dart, A. E., The estimation of bile acids in bile, *J. Biol. Chem.*, 1920–21, xlv, 415.
38. Sheard, C., Baldes, E. J., Mann, F. C., and Bollman, J. L., Spectrophotometric determinations of bilirubin, *Am. J. Physiol.*, 1926, lxxvi, 577.
39. Smyth, F. S., and Whipple, G. H., Bile salt metabolism. I. Influence of chloroform and phosphorus on bile fistula dogs, *J. Biol. Chem.*, 1924, lix, 623.
40. Szilard, P., Eine neuere kolorimetrische Methode zur quantitativen Bestimmung der gallensäuren Salze im Blute, *Biochem. Z.*, 1926, clxxiii, 440.
41. Tashiro, S., Determination of bile salts in the blood. Preliminary report, *Cincinnati J. Med.*, 1923, iv, 197.
42. von Udranszky, L., Ueber Furfurolreactionen, *Z. physiol. Chem.*, 1888, xii, 355, 377.
43. Whipple, G. H., Origin and significance of constituents of the bile, *Physiol. Rev.*, 1922, ii, 440.
44. Wu, H., A modification of Duboscq-Bellin colorimeter for bicolorimetric work, *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 111.

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THE CHEMISTRY OF JAFFE'S REACTION FOR CREATININE.

IV. A COMPOUND OF CREATININE, PICRIC ACID, AND SODIUM HYDROXIDE.

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In experiments described in a previous publication (1), it was found that both the creatinine and the picric acid involved in Jaffe's reaction could, after acidification with acetic acid, be recovered unchanged. It was also found that, when a fairly concentrated solution of creatinine, sodium picrate, and sodium hydroxide is acidified with hydrochloric acid, there is precipitated a red tautomer of creatinine picrate.

In some recent unpublished experiments, it has been found that the red color obtained when a barbituric acid solution is treated with sodium picrate and sodium hydroxide is not due to the formation of picramic acid, as Dox believed (2) but that, after acidification with acetic acid, the picric acid can be recovered unchanged. Addition of hydrochloric acid to the alkaline reaction mixture does not yield a red precipitate. However, upon treatment of the red alkaline solution with several volumes of alcohol, a red precipitate is obtained. This appears to be a compound of 2 molecules of picric acid, 3 of barbituric acid, 9 of sodium hydroxide, and 1 or 2 of water. These experiments will be described in another publication.

It seemed to be of interest to see whether a similar compound could be obtained from creatinine. After a preliminary experiment had shown that the addition of alcohol to the mixture of creatinine, sodium picrate, and sodium hydroxide did, indeed, yield a red precipitate, a larger quantity was prepared. This material, denoted Preparation I in Table I, was dissolved in water and reprecipitated with alcohol to yield Preparation II. The

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composition of these, and subsequent preparations, is shown in Table I. It is evident from the data for Preparations I and II, and for Preparations IV and VI, that the composition of the precipitate is not essentially affected by solution and reprecipitation. Absolute constancy of composition in the different preparations is not to be expected. The aqueous solution contains a mixture of

TABLE I.

Analyses of New Compounds of Creatinine with Sodium (or Lead) Picrate, Sodium (or Lead) Hydroxide, and Water.

	Picric acid.	Sodium.	Non-picric N.	Total N.
	per cent	*per cent*	*per cent*	*per cent*
Calculated for compound of 2 molecules of creatinine, 1 of picric acid, 3 of sodium hydroxide, 3 of water	36.4	11.0	13.4	20.0
Preparation I.	35.2	11.9		
" II (Preparation I dissolved and reprecipitated)	36.2	11.5	13.8	
" III	36.3	11.7	13.9	
" IV	37.1	11.9	13.2	
" V	36.3	10.6		
" VI (Preparation IV, redissolved and reprecipitated)	36.3	12.4	13.7	
" VII	36.3	12.0	13.5	19.8
		Lead.		
Calculated for compound of 2 molecules of creatinine, 1 of picric acid, 3 of lead hydroxide, 3 of water	18.6	50.4	6.81	10.2
Preparation VIII	16.1	56.6	5.91	
" IX	16.9	56.4	6.60	9.32

sodium hydroxide, sodium picrate, creatinine, the new compound, and, in all probability, at least one other (see the following paragraph).

The substance is an orange, hygroscopic powder. It darkens when heated but does not melt, even at 265°. When heated at 100°, or even at 80°, *in vacuo*, it loses a small quantity of water. But decomposition also occurs, as is evidenced by the darkening and by the lessened amount of picric acid to be obtained after

solution and acidification. The substance is readily soluble in water, giving an orange-red solution. *The color is not nearly deep enough to account for the red color of Jaffe's reaction. It is slightly intensified by the addition of more sodium picrate and more sodium hydroxide but, even then, the color is a pale yellow orange and not the deep red-orange obtained with the equivalent amount of creatinine. The full color value of the creatinine can be obtained only if the substance is first dissolved in dilute acetic acid. Therefore, the compound described in this paper is not the substance responsible for Jaffe's reaction. That may be a similar compound containing more sodium picrate and more sodium hydroxide.*

The results of the analyses correspond quite closely with those calculated for a compound consisting of 2 molecules of creatinine, 1 of picric acid, 3 of sodium hydroxide, and 3 of water. The sodium content is, however, always slightly greater than that calculated. The significance of this is not at all clear.

When a solution of the new substance is treated with basic lead acetate solution, a red precipitate is obtained. When filtered out, washed with water, and dried over sulfuric acid, *in vacuo*, this forms a red powder. Upon analysis, it appears that it is a compound of 2 molecules of creatinine, 1 of picric acid, 3 of lead hydroxide, and 3 of water. As in the case of the sodium compound, the base is present in excess. As might be expected, the excess of base is greater in the lead compound than it is in the sodium compound. Presumably, because of the great equivalent weight of the excess lead, the observed values for the other constituents are considerably lower than those calculated.

When this lead compound is treated with a hot mixture of equal volumes of concentrated sulfuric acid and water, a distinct effervescence may be observed. If the supernatant liquid be then diluted, neutralized with sodium hydroxide, and treated with nitron, only a trace of a precipitate is produced. The picric acid has been destroyed. If, instead of neutralizing the liquid, hydrochloric acid and tin are added and the mixture is used for the determination of total nitrogen, it is found that not quite one-ninth of the total nitrogen has been lost. The same result is obtained when the lead compound is dissolved in concentrated hydrochloric acid. (See Table II.)

When the sodium compound is similarly treated with concen-

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trated hydrochloric acid or with hot 65 per cent sulfuric acid, there is the same destruction of the picric acid but there is no effervescence and there is no loss of nitrogen. In other experiments, the sodium compound was dissolved in concentrated sulfuric acid, which was then heated just to boiling, was then cooled, diluted, and treated with hydrochloric acid and tin in the usual manner. Under these conditions, there was a loss of nitrogen amounting to not quite two-ninths of the total nitrogen. The lead compound, on the other hand, loses only one-seventh of its nitrogen when treated in this manner.

No attempt has been made to formulate the manner in which the creatinine, the sodium (or lead) picrate, the sodium (or lead)

TABLE II.

Effect of Concentrated Acids upon Apparent Nitrogen Content of the New Compounds.

	N calculated.	N found according to:			
		Procedure A.	Procedure B.	Procedure C.	Procedure D.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
In sodium compound.....	20.0	19.8	19.8	19.7	16.0
" lead "	10.2	9.32	8.50	8.32	7.96

hydroxide, and the water are combined. That must await the attempted preparation of similar, related compounds and the study of the decomposition products of the new substances. It is believed that the tenacity with which the water is held, the destruction of picric acid upon heating, the loss of nitrogen upon treatment with concentrated acids, and the excess of base over that calculated are all of significance.

EXPERIMENTAL.

Preparation.

Details of Preparation VII.—11.0 gm. of creatinine picrate were dissolved in 200 cc. of hot water. 3.65 gm. of creatinine were added and the mixture was then cooled. When cold, a saturated solution of sodium hydroxide was slowly added. 3

cc. sufficed to bring the picrate into solution but 9 cc. more, or a total of 12 cc., were added. After 10 minutes, the mixture was slowly run into 1600 cc. of absolute alcohol, with continuous stirring. (It is important that the solution be added *slowly*, with continuous stirring. Otherwise, the substance is precipitated as a gum.) The flocculent precipitate was filtered out, with suction, washed with a little alcohol, dissolved in water, and precipitated as before. The process of filtration, solution, and reprecipitation was again repeated. After filtering off this precipitate, it was dried, *in vacuo*, over sulfuric acid. The yield was 18.6 gm. The calculated yield is 20.3 gm.

Analyses.

Picric Acid.—The material was dissolved in 150 cc. of 1 per cent acetic acid, and the solution was heated to boiling and precipitated with nitron (3). The nitron picrate was filtered on a Gooch crucible, dried, and weighed.

Sodium.—The filtrate from the nitron picrate was evaporated to about 50 cc. and 1 or 2 cc. of nitric acid were then added. The nitron nitrate that crystallized out on cooling was filtered out and washed with a little water. The filtrate, after the addition of 5 to 10 drops of sulfuric acid was evaporated in a platinum crucible, then ignited, and weighed. In many cases, the identity of the residue as sodium sulfate was checked by solution, precipitation with barium chloride, and weighing as barium sulfate. In other cases, the original material was weighed into the platinum crucible, 1 or 2 cc. of sulfuric acid were added, and the mixture was evaporated, ignited, and weighed.

Lead.—The filtrate from the nitron picrate was treated with hydrogen sulfide; the lead sulfide was filtered out, washed, oxidized with nitric and sulfuric acids, and finally weighed as lead sulfate.

Non-Picric Nitrogen.—The acetic acid solution of the original material was treated with the quantity of nitron calculated to be just sufficient to combine with the picric acid present, as previously determined on another sample. The nitron picrate was filtered out and washed and the filtrate was used for the usual nitrogen determination. Since the errors involved are cumulative in one direction, the values tend to be high.

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Total Nitrogen.—This was determined by the Kjeldahl method. The acid digestions were carried out in several different ways. Procedure A. The material was dissolved in dilute acetic acid. Hydrochloric acid and tin were then added, the mixture heated until colorless, and the digestion then completed with sulfuric acid and copper sulfate in the usual manner. Procedure B. The material was dissolved in concentrated hydrochloric acid, tin was added, and the mixture digested until colorless. After the addition of sulfuric acid and copper sulfate, the determination was completed in the usual manner. Procedure C. The material was dissolved in a boiling mixture of 20 cc. of concentrated sulfuric acid and 20 cc. of water. It was then diluted, hydrochloric acid and tin were added, and the determination completed as in Procedures A and B. Procedure D. The material was dissolved in concentrated sulfuric acid which was then heated just to boiling. It was then cooled, water, hydrochloric acid, and tin were added, and the digestion completed.

Formation of Creatinine Picrate from the New Compound.

1.60 gm. of Preparation VII were dissolved in 1 per cent acetic acid. After the addition of 0.5 gm. of picric acid, the mixture was heated to boiling and was then allowed to cool. The crystals that separated were filtered out and recrystallized. They then melted at 221° and contained 66.1 per cent picric acid. Calculated for creatinine picrate, 67 per cent. The yield was 1.50 gm. Allowing for the solubility of the creatinine picrate (4), the total yield was 1.76 gm. The calculated amount is 1.71 gm.

Effect of Heating on the Picric Acid Content.

0.4728 gm. of Preparation III was heated in an Abderhalden tube at 100° and about 40 mm. for 1.5 hours. The material then had a dark red color. It had lost 30.9 mg. or 6.54 per cent. This corresponded to something more than 2 molecules of water. The residue was dissolved in dilute acetic acid and precipitated with nitron in the usual manner. 0.3956 gm. of nitron picrate was obtained. This was the equivalent of 35.4 per cent of picric acid in the original material, instead of the 36.3 per cent found in the unheated material. The filtrate, on cooling, deposited a small quantity of a brown precipitate, apparently nitron picramate.

Effect of Concentrated Acids.

6.07 gm. of a mixture of Preparations III and IV were dissolved in 200 cc. of water, to which 15 cc. of concentrated hydrochloric acid were then added. Upon extraction with benzene in a continuous extraction apparatus, only 0.1604 gm. of material soluble in benzene was obtained, instead of the 2.23 gm. to be expected. Not even all of this small quantity was picric acid for it was not all soluble in water. Its nature was not further investigated.

Color Development from New Substance with Added Sodium Picrate and Sodium Hydroxide.

Portions of 13.8 mg. each, equivalent to 5 mg. of creatinine, of Preparation VII were weighed into a series of small beakers. These were treated as follows: Sample 1. Dissolved in 10 cc. of water, 2 drops of acetic acid, heated to boiling, and allowed to cool. Sample 2. Dissolved in 10 cc. of water, 2 drops of acetic acid, and allowed to stand. Sample 3. Dissolved in 5 cc. of water and 10 cc. of 1 per cent picric acid. Sample 4. Dissolved in 4.1 cc. of water and 0.9 cc. of 0.1075 N H_2SO_4. (This is about 10 per cent in excess of the equivalent of the sodium content of the sample.) Sample 5. Remained undissolved.

Sample 1 became yellow on warming; Samples 2, 3, and 4 changed from orange to yellow within an hour but all the mixtures were allowed to stand overnight. 10 cc. of 1 per cent picric acid were then added to each, except Sample 3, and they were all diluted to 20 cc. with water. 5 cc. of 10 per cent NaOH were then added and, after standing 10 minutes, the mixtures were diluted to 200 cc. and the colors were then compared with that of a similar mixture containing 5 mg. of creatinine. The apparent amounts of creatinine found were:

	mg.
Sample 1	4.85
" 2	4.98
" 3	4.22
" 4	4.98
" 5	0.00

The mixture in which the new substance was treated directly with picric acid and alkali did not develop a red-orange color and the diluted material was yellow and not orange.

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Test for Alcohol in the Sodium Compound.

3.09 gm. were dissolved in 200 cc. of water and 1 cc. of H_2SO_4. The mixture was distilled slowly, about 30 minutes being required for 25 cc. Determinations of the specific gravity and the iodoform test indicated the absence of alcohol.

The creatinine used in these experiments was made from creatine generously supplied by the Valentine's Meat Juice Company.

BIBLIOGRAPHY.

1. Greenwald, I., and Gross, J., *J. Biol. Chem.*, 1924, lix, 601.
2. Dox, A. W., *Z. physiol. Chem.*, 1925, cl, 118.
3. Busch, M., and Blume, G., *Z. angew. Chem.*, 1908, xxi, 354.
4. Greenwald, I., *Biochem. J.*, 1926, xx, 665.

ON THE STATE OF PLASMA CALCIUM IN PARATHYROIDECTOMIZED DOGS.*

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(Received for publication, March 14, 1928.)

In a recent publication (1) it was shown that after parathyroidectomy dogs experience tetany when the plasma calcium concentration falls and the inorganic phosphorus concentration increases until the two are approximately equal, or, until the Ca:P ratio is 1. Since that paper was prepared it has been found that this relationship holds good only for the earlier postoperative periods, for, after an animal attains a state of "latency" it may escape tetany for as much as 10 days or longer, even though the ratio is continuously as low as 0.6. It is apparent, therefore, that in latent tetany the animal must undergo some sort of adaptation that enables it to escape tetany.

In view of the various conflicting reports as to alterations in the state of calcium in the blood, it has seemed advisable to investigate the question of possible alterations in diffusibility in latency, in the hope of throwing some light on the mechanism of adaptation.

It has been assumed that animals in latent tetany have undergone a stimulation of remnants of parathyroid tissue to such a degree that this residual tissue can function to some extent in place of the main bodies. Possibly this explanation will serve in those cases in which there is partial or complete restoration of the normal proportions and concentrations of calcium and phosphorus. But in this laboratory it has been found that in the majority of such cases there is no such restoration. Therefore adaptation involves some mechanism not yet determined.

* A part of the expenses of this investigation was borne by a grant from the Research Committee of Phi Rho Sigma Medical Fraternity.

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Rona and Takahashi (2), by the method of compensation dialysis, determined that about 75 per cent of serum calcium was in diffusible form. Cruikshank (3), by the same method, found 60 to 70 per cent diffusible, and after parathyroidectomy about 94 per cent diffusible; therefore the most rapid reduction occurred in the non-diffusible fraction. Von Meysenbug (4) and his associates found similar percentages in normal animals but concluded that both fractions were equally reduced in tetany. However, most of these observations were made within 2 to 4 days after operation. In one case, 14 days after operation, the diffusible percentage appears consistently slightly higher than the average over several days at earlier stages. Their conclusion is in agreement with that of MacCallum, Lambert, and Vogel (5). Salvesen and Linder (6) found 55 to 70 per cent of calcium diffusible and believed that the loss in tetany affected this fraction primarily, but that, as a result of disturbed equilibrium between the two portions, the non-diffusible might be affected in varying degrees, the latter acting as a reserve for calcions. They believed the symptoms of parathyroid deficiency to be due to hypocalcionemia. Hence the actual proportions found at any given time might vary considerably.

Loeb (7) found 55 to 75 per cent diffusible calcium. Cameron and Moorhouse (8), by simultaneous determinations of calcium in plasma and cerebrospinal fluid, concluded that from 42 to 73 per cent was diffusible, with an average of 53 per cent; while in tetany, from 70 to 100 per cent was diffusible. Moritz (9) believed that both fractions were reduced in tetany but that the diffusible was most affected. Updegraff, Greenberg, and Clark (10) concluded from a study of normal human serum from both sexes and that of rabbits, dogs, and calves, that the diffusible calcium varies between 40 and 60 per cent and that the absolute amount of this fraction is more constant.

Liu (11), in a study of infantile and adult idiopathic tetany, concluded that hypocalcemia in this condition affects mainly the diffusible fraction, which he found amounted to 40 to 60 per cent in normal subjects. He further found, in agreement with others that parathormone produced relatively a greater increase in this portion in both normal and tetanic subjects.

EXPERIMENTAL.

Eight dogs have been studied in this connection but the history is complete in only four so the discussion will be confined mainly to the results obtained with these. Observations extended over a period of several days before operation. No significant variations in the proportions or absolute concentrations of the two fractions occurred in any normal dog.

Diffusible calcium was separated by a combination of the methods of Moritz (9) and of Updegraff, Greenberg, and Clark (10) with only minor modifications. The calculations introduced by

the latter were employed throughout. Collodion tubes were numbered and a careful record kept of the results obtained with each one, so that it was possible to discover and eliminate faulty tubes. In the earlier experiments, a water suction pump was employed for producing negative pressure but later a mercury siphon was constructed. The apparatus was so arranged that six determinations could be made simultaneously. Enough heparinized plasma was usually obtained so that it was possible to make one or more duplicate determinations, either with the same tube on successive runs or with separate tubes. In this way the relative permeability of different tubes was standardized. Each run was continued for at least 5 hours, many for a much longer period. But in the latter case results checked well with those obtained from the minimum period. After some preliminary attempts, it was found possible to duplicate results within $\pm$ 4 per cent.

Both total and *diffused* calcium concentrations were determined by the method of Clark and Collip (12), with the minor modifications described elsewhere (1). It was found, however, that if precipitation with ammonium oxalate was prolonged for several hours instead of 30 minutes, slightly, but consistently higher results were obtained. Permanganate solutions were standardized for each determination.

For a small number of determinations of diffusible phosphorus, the method of Briggs (13) was used; for a similar number, that of Fiske and Subbarow (14). In confirmation of the results of others (15, 16), it was found that the phosphorus of normal dog plasma was completely diffusible and there was no alteration at any time after parathyroidectomy.

Among normal animals, diffusible calcium ranged between 40 and 70 per cent. The maximum range in any one animal was from 39 to 52 per cent on successive days. It is possible that this one figure—39 per cent— is an error since all other determinations on this dog ranged between 47 and 52 per cent, with practically constant total calcemia. In all other animals the range of variation during the normal period was within experimental error. There was thus a wide range of variation among different animals but fairly constant proportions in the same one.

Table I shows the condensed results. It will be seen that in

TABLE I.

	Total Ca.	Diffusible Ca.	Non-diffusible Ca.	Diffusible Ca.	Inorganic P.	Ca:P	Remarks.
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	*mg. per 100 cc.*		
Dog 51.							
Average for 14 days preoperative.	10.40	4.65	5.75	45	5.99	1.7	
2 days postoperative.	7.58	2.40	5.18	32	7.02	1.1	No tetany.
3 " "	6.35	2.80	3.55	44	7.21	0.88	Tetany; N.*
5 " "	6.80	3.40	3.40	50	11.0	0.61	Severe tetany; Pt.*
16 " "	8.38	4.96	3.42	59	8.07	1.03	No tetany.
19 " "	7.80	3.68	4.12	47	9.56	0.8	Tremors, spasticity.
22 " "	6.33	3.85	2.48	61	7.69	0.8	Tetany.
33 " "	6.54	3.20	3.34	48	8.50	0.8	Violent tetany.
43 " "	9.05	4.61	4.44	51	5.71	1.6	Cachexia; Pt.
44 " "	9.78	5.56	4.22	57	5.65	1.7	
59 " "	6.42	5.24	1.18	78	5.40	1.18	61st day tetany; died.
Dog 52.							
Average for 10 days preoperative.	13.49	5.82	7.67	43	7.28	1.85	
4 days postoperative.	9.05	2.67	6.38	30	8.00	1.1	Tetany; glucose.
5 " "	10.35	5.21	5.14	50	7.57	1.3	Very mild tremors.
12 " "	8.24	3.64	4.60	44	6.66	1.2	No tetany.
17 " "							Death from distemper.
Dog 56.							
Average for 5 days preoperative.	14.16	9.91	4.25	70			
3 days postoperative.	6.42	5.33	1.09	83	7.40	0.86	Tetany; glucose.
6 " "	7.11	3.35	3.76	47	7.17	0.99	Severe tetany; Pt.
18 " "	5.70	5.57	0.13	96	6.72	0.84	" " glucose.
40 days postoperative.	6.42	4.34	2.08	67	5.67	1.1	Mild tetany; glucose.
52 " "	8.43	3.38	4.45	41	6.15	1.3	No tetany.

40 days postoperative.	6.42	4.34	2.08	67	5.67	1.1	Mild tetany; glucose.
52 " "	8.43	3.38	4.45	41	6.15	1.3	No tetany.
52 " "	5.08	3.40	1.68	67	5.67	0.89	Severe tetany.
59 " "	4.15	2.31	1.84	56	6.20	0.6	No tetany.
68 " "							Died in coma.
Dog. 60.							
Average for 13 days preoperative.	11.93	6.61	5.32	55	4.57	2.4	
4 days postoperative.	11.51	5.92	5.59	51	4.37	2.6	No tetany.
9 " "	10.40	5.13	5.27	49	5.03	2.0	" "
25 " "	7.48	6.00	1.48	80	4.08	1.8	Violent tetany.
Dog 63.							
Average for 4 days preoperative.	15.71	11.4	4.30	72	5.59	2.7	
15 days postoperative.							First known attack of tetany; no treatment.
25 " "	8.85	6.16	2.69	69	Lost.		Severe tetany.

* N., no treatment; Pt., parathormone.

case of Dogs 51 and 52, there was an appreciable decrease in both absolute concentration and percentage of diffusible calcium, coinciding with the first attack of tetany, but gradual restoration of the percentage and even of the absolute amount within a few days. In case of Dog 60, the changes were less pronounced. It seems probable that not all the parathyroid tissue was removed from this dog, since only one attack of tetany has occurred—25 days after operation—and the dog has shown no further disturbance in 45 days of postoperative observation. Frequent examination of blood shows that calcium and phosphorus concentrations have been in normal proportions since 2 days after this attack.

Also, in case of Dog 56, there was an early decrease in the absolute concentration of diffusible calcium but never complete restoration although the percentage increased greatly at times. But both amount and percentage showed greater fluctuations than in any of the other animals. Thus the first attack of tetany occurred in two cases when the percentage and absolute concentration were low, while in the others, the first attack occurred at lower amounts but high percentages.

So far as our observations extend, the tendency to instability between the two fractions seems to be a characteristic feature of prolonged parathyroid deficiency. It is obvious, in spite of Liu's contention, that in these experimental animals no relationship can be established between the incidence of tetany and either the absolute amount or the percentage of diffusible calcium. Nor does the ratio between this fraction and inorganic phosphemia show any constant relation to tetany. It can only be stated that in the early postoperative period there was a tendency to relative and absolute diminution in the concentration of diffusible calcium and a pronounced degree of instability thereafter, possibly some tendency to increase the percentage.

In regard to the question of adaptation, it may be noted that Dog 56, after a severe attack of tetany on the 52nd day, showed a ratio much less than unity for nearly 2 weeks without any sign of tetany. This animal died in a state of profound depression without any further attacks.

That Dog 52 suffered one attack when the Ca:P ratio was 1.3, may have been due to intestinal parasites found at autopsy. The behavior of Dog 60 cannot be accounted for entirely satisfactorily

at present. These two instances complete a total of six out of 143 observations of tetany with a ratio definitely above 1.

In case of two other animals not included in Table I, the above mentioned adaptation was quite pronounced. Dog 36 survived 97 days after operation. During the last 4 weeks there were several attacks but there were also periods of as much as 10 days during which the Ca:P ratio ranged between 0.7 and 0.9 when no attacks occurred. During the first 5 weeks a ratio of 1 invariably presaged a severe attack. In case of Dog 43, which survived 214 days, there was likewise a period of more than 4 months during which low ratios persisted for 5 to 10 days without tetany. On the 198th day this dog gave birth to three puppies but for 12 days thereafter was free from all signs of tetany, with a calcemia of 6 to 7 mg. and inorganic phosphemia of 8 to 9 mg. Tetany did occur on the 13th day, when the dog received 40 units of parathormone. 3 days later the animal died in a period of depression with no evidence of any further attacks.

It has been suggested that parathyroid deficiency results first in a decrease in the diffusible fraction of calcium, with a loss of equilibrium that eventually leads to transfer from the non-diffusible fraction and consequent reduction in that also. If this should be true, it would explain the instability apparent here, since the determined percentage would depend on the particular stage in the process of transfer at which the observation was made.

It has been questioned whether determinations of diffusibility of calcium by means of collodion membranes are of any significance in connection with the physiological processes involved in calcium metabolism. It must be acknowledged that *this* investigation has yielded no evidence that would help to decide this point. The facts can only be stated as they occur. However, the relative stability always found in the normal animal is strongly suggestive of an analogy between the collodion membrane and the animal membrane in this connection. At least, parathyroidectomy leads to changes in the blood that are detectable by this means.

SUMMARY.

1. Determination of diffusible plasma calcium in parathyroidectomized dogs shows that certain individuals undergo a

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pronounced diminution of one fraction or the other, but there is no constancy of response among different animals, tetany occurring both at high and low concentrations and proportions. There is less stability between the two fractions than in the normal animal.

2. The incidence of tetany in the early postoperative period is associated with a change in the proportions of calcium and inorganic phosphorus sufficient to lower the Ca:P ratio to 1 or less. But in latent tetany there is an adaptation that enables the animal to survive a low ratio without tetany, for long periods.

BIBLIOGRAPHY.

1. Reed, C. I., Lackey, R. W., and Payte, J. I., *Am. J. Physiol.*, 1928, lxxxiv, 176.
2. Rona, P., and Takahashi, D., *Biochem. Z.*, 1911, xxxi, 336.
3. Cruikshank, E. W. H., *Brit. J. Exp. Path.*, 1923, iv, 213.
4. von Meysenbug, L., Pappenheimer, A. M., Zucker, T. F., and Murray, M. F., *J. Biol. Chem.*, 1921, xlvii, 529. von Meysenbug, L., and McCann, G. F., *J. Biol. Chem.*, 1921, xlvii, 541.
5. MacCallum, W. G., Lambert, R. A., and Vogel, K. M., *J. Exp. Med.*, 1914, xx, 149.
6. Salvesen, H. A., and Linder, G. C., *J. Biol. Chem.*, 1923–24, lviii, 617, 635.
7. Loeb, R. F., *J. Gen. Physiol.*, 1923–24, vi, 453.
8. Cameron, A. T., and Moorhouse, V. H. K., *J. Biol. Chem.*, 1925, lxiii, 687.
9. Moritz, A. R., *J. Biol. Chem.*, 1925, lxiv, 81; 1925, lxvi, 343.
10. Updegraff, H., Greenberg, D. M., and Clark, G. W., *J. Biol. Chem.*, 1926–27, lxxi, 87.
11. Liu, S. H., *Chinese J. Physiol.*, 1927, i, 331; *J. Clin. Inv.*, 1928, v, 259, 277.
12. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, lxiii, 461.
13. Briggs, A. P., *J. Biol. Chem.*, 1924, lix, 255.
14. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, lxvi, 375.
15. Neuhausen, B. S., and Pincus, J. B., *J. Biol. Chem.*, 1923, lvii, 99.
16. Grollman, A., *Am. J. Physiol.*, 1925–26, lxxv, 287; *J. Gen. Physiol.*, 1926, ix, 813; *J. Biol. Chem.*, 1925, lxiv, 141; 1927, lxxii, 565.

CONFIGURATIONAL RELATIONSHIPS OF 2-HYDROXY-VALERIC AND LACTIC ACIDS.

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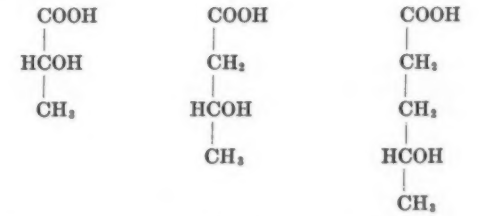
(Received for publication, March 10, 1928.)

Until recently the configuration of 2-hydroxypropionic (lactic) acid only had been established by direct chemical methods. The conclusions regarding the configurations of the higher monohydroxy acids were based on indirect methods and such conclusions are never as convincing as those arrived at by direct methods. Our first efforts in this direction resulted in correlating the configurations of 3-hydroxybutyric[1] and 4-hydroxyvaleric acids[2] with that of lactic acid.



Levo-lactic acid.	Levo-3-hydroxybutyric acid.	Levo-4-hydroxyvaleric acid.
I.	II.	III.

This beginning proved very fortunate, for it permitted us to establish the configurations of methylethyl and methylpropyl carbinols.[3] The knowledge of the configurations of these two car-

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxv, 49; 1926, lxvii, 329. Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1926, lxviii, 415.

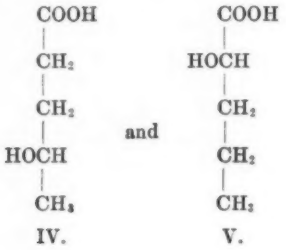
[2] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165, 569.

[3] Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 591. Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1926-27, lxxi, 465.

binols made it possible to establish the configurations of 2-hydroxybutyric and 2-hydroxyvaleric acids. The work on the configuration of 2-hydroxybutyric acid has been made the topic of a previous communication[4] and the work on the configuration of 2-hydroxyvaleric acid is presented here.

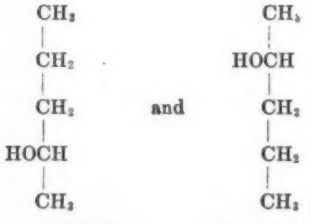
The reasoning which led to the conclusion regarding the configuration of 2-hydroxyvaleric acid is analogous to that which served to elucidate the configuration of 2-hydroxybutyric acid.

Taking two hydroxy acids with the hydroxyls on the same side of the carbon chain, but allocated at different distances from the carboxyl, such as



IV. V.

and in each case reducing the carboxyl group to a methyl group, two carbinols which are enantiomorphous to each other will be obtained.



Dextro-carbinol from (IV). VI.

Levo-carbinol from (V). VII.

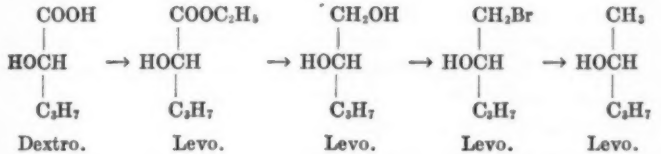
Hence if a given optically active 2-hydroxyvaleric acid is converted into levo-methylpropyl carbinol, this acid has the hydroxyl

[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxiv, 343.

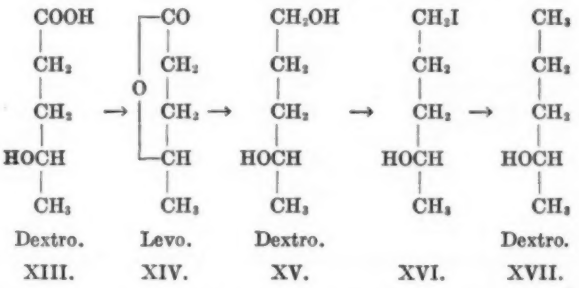
on the same side of the carbon chain as that of the 4-hydroxyvaleric acid which leads to a dextro-methylpropyl carbinol, namely the dextrorotatory form.

In our case, dextro-2-hydroxyvaleric acid on reduction yielded levo-methylpropyl carbinol; hence in this acid the hydroxyl is allocated on the same side of the chain as in that 4-hydroxyvaleric acid which on reduction gives dextro-methylpropyl carbinol. That acid is dextro-4-hydroxyvaleric acid. Hence in dextro-2-hydroxyvaleric acid the hydroxyl is on the same side of the chain as in dextro-4-hydroxyvaleric acid.

The set of reactions which led to the correlation of dextro-2-hydroxyvaleric acid with levo-methylpropyl carbinol is the following:



For comparison we give the set of reactions leading from dextro-4-hydroxyvaleric acid to dextro-methylpropyl carbinol.



An important practical significance of this conclusion lies in the fact that it furnishes additional evidence in support of the validity of the indirect method for correlating the configurations of substituted aliphatic acids. The dextro-2-hydroxyvaleric acid on passing to its anion changed its rotation to the left, and, according to the rule

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developed in this laboratory, the acid should belong to the l series, and now it has been shown by the direct method that it does belong to the l series.

EXPERIMENTAL.

Relationship of 2-Hydroxy-n-Valeric Acid to 1,2-Dihydroxypentane.

Dextro-2-Hydroxy-n-Valeric Acid.—The inactive acid was obtained from 2-bromo-*n*-valeric acid. 181 gm. of 2-bromo-*n*-valeric acid were added to 800 cc. of water containing 138 gm. of potassium carbonate. The solution was heated under a reflux condenser on a boiling water bath for 8 hours. It was then cooled, a solution of 50 gm. of concentrated sulfuric acid in 100 cc. of water was added carefully, and the solution was extracted with ether in a continuous ether extractor. The ether extract was dried over sodium sulfate and the ether removed under reduced pressure. The acid was dissolved in warm acetone and neutralized with 1 equivalent of brucine. The brucine salt, which crystallized readily, was recrystallized repeatedly from acetone. It was then decomposed with ammonia in the usual way and the ammonium salt was converted into the barium salt. In water the barium salt had the following rotation.

$$[\alpha]_D^{25} = \frac{-0.31^\circ \times 100}{2 \times 3.16} = -4.9^\circ.$$

The rotation of the free acid was obtained by dissolving 1.000 gm. of the barium salt in slightly more than 1 equivalent of concentrated hydrochloric acid and diluting to 5 cc. with water.

$$[\alpha]_D^{25} = \frac{+0.38^\circ \times 100}{2 \times 12.7} = +1.5^\circ.$$

Levo-Ethyl-2-Hydroxy-n-Valerate.—To 40 gm. of thoroughly dried barium-2-hydroxy-*n*-valerate ($[\alpha]_D^{25} = -4.9^\circ$) suspended in 100 cc. of absolute alcohol a solution of 12 gm. of concentrated sulfuric acid in 50 cc. of absolute alcohol was added slowly. The mixture was vigorously agitated during the addition of the acid solution. It was then refluxed for 5 hours and allowed to stand overnight. The excess acid was neutralized with solid potassium carbonate, dry ether was added, and the solution filtered from salts. After

drying over anhydrous sodium sulfate the ether was removed and the ester was distilled under reduced pressure. It distilled at 81°, p = 20 mm. The yield was 18 gm. It analyzed as follows:

7.080 mg. substance: 14.840 mg. CO_2 and 6.165 mg. H_2O.
$C_7H_{14}O_3$. Calculated. C 57.49, H 9.65.
Found. " 57.15, " 9.74.

In a 1 dm. tube the optical rotation without solvent was $\alpha_D^{20} = -5.05°$.

Levo-1,2-Dihydroxypentane.—This glycol was obtained on reduction of levo-ethyl-2-hydroxy-*n*-valerate ($\alpha_D^{20} = -4.80°$ in a 1 dm. tube) with sodium and glacial acetic acid in the apparatus described by Levene and Allen.[5] The procedure for the reduction was the same as that described previously for the reduction of other hydroxy acids.[6] The glycol distilled at 78–81°, p = 1 mm. It analyzed as follows:

3.742 mg. substance: 8.045 mg. CO_2 and 3.660 mg. H_2O.
$C_5H_{12}O_2$. Calculated. C 57.69, H 11.53.
Found. " 58.62, " 10.94.

In absolute alcohol it rotated the plane of polarized light as follows:

$$[\alpha]_D^{20} = \frac{-1.53° \times 100}{2 \times 7.80} = -9.8°.$$

Di-(Phenylurethane) of Levo-1,2-Dihydroxypentane.—1 part of levo-1,2-dihydroxypentane ($[\alpha]_D^{20} = -9.8°$) and 2.33 parts of phenylisocyanate were heated on the steam bath for 15 minutes. When the reaction product was allowed to stand overnight, it crystallized. It was recrystallized several times from dilute alcohol. It melted at 110–113° and analyzed as follows:

0.0500 gm. substance: (Kjeldahl) 2.95 cc. 0.1 N acid.
$C_{19}H_{22}O_4N_2$. Calculated. N 8.18.
Found. " 8.26.

In absolute alcohol it rotated the plane of polarized light as follows:

$$[\alpha]_D^{20} = \frac{-1.33° \times 100}{2 \times 5.44} = -12.2°$$

[5] Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 433.
[6] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165. Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 593.

XUM

Preparation of Dextro-1,2-Dihydroxypentane from d,l-1-Chloro-2-Hydroxypentane.

d, l-1-Chloro-2-Hydroxypentane.—This substance was obtained on condensation of chloroacetaldehyde with propyl magnesium bromide.

A mixture of 150 gm. of chloroacetal and 90 gm. of anhydrous oxalic acid was slowly distilled. The fraction which distilled at 88–110° was collected and diluted with an equal volume of dry ether. The solution was shaken with 80 gm. of powdered calcium chloride for 4 hours. It was then filtered and the filtrate dropped into a well cooled Grignard reagent prepared from 25 gm. of magnesium, 125 gm. of propyl bromide, and 400 cc. of dry ether. The reaction product was allowed to stand overnight and then decomposed with ice and 1:1 hydrochloric acid in the usual manner. The acid solution was extracted with ether several times. The combined ether extracts were washed successively with dilute hydrochloric acid, water, 10 per cent potassium carbonate solution, and water. The ether solution was dried over anhydrous potassium carbonate for 2 hours and then over sodium sulfate overnight. The ether was removed and the remaining product fractionally distilled. A fraction which distilled at 59–62°, p = 14 mm., was collected and analyzed as follows:

3.490 mg. substance: 6.180 mg. CO_2 and 2.800 mg. H_2O.
0.1122 gm. " : 0.1294 gm. AgCl.
$C_5H_{11}OCl$. Calculated. C 48.94, H 9.06, Cl 28.95.
Found. " 48.29, " 8.97, " 28.53.

Chloromethylpropyl Ketone (1-Chloropentanone-(2)).—The chloropentanol obtained as described above was oxidized with potassium dichromate and sulfuric acid. The reaction mixture was steam-distilled and the chloroketone extracted from the distillate with ether. The ether solution was dried over sodium sulfate. After removal of the ether the chloroketone was distilled. It distilled at 55–57°, p = 15 mm. It readily formed a bisulfite compound. It analyzed as follows:

0.1110 gm. substance: 0.1316 gm. AgCl.
C_5H_9OCl. Calculated. Cl 29.43.
Found. " 29.33.

Hydroxymethylpropyl Ketone (Pentanol-(1)-one-(2)).—A mixture of 50 gm. of chloromethylpropyl ketone, 140 gm. of dried potassium formate, and 100 cc. of dry methyl alcohol was heated under a reflux condenser on a boiling water bath overnight. The reaction mixture was cooled, dry ether was added, and the solution filtered. After drying the solution over sodium sulfate, the solvent was removed and the hydroxyketone was distilled under reduced pressure. The fraction which distilled at 62–64°, p = 18 mm., was collected and employed for reduction to the glycol.

Dextro-1,2-Dihydroxypentane.—To an actively fermenting mixture of 450 gm. of cane sugar, 450 gm. of bakers' yeast, and 4500 cc. of water were added 45 gm. of hydroxymethylpropyl ketone. The reaction mixture was allowed to stand 6 days and then worked up in the usual manner.[7] The glycol thus obtained was redistilled from a flask provided with a Vigreux column. It distilled at 97–99°, p = 13 mm. It analyzed as follows:

5.930 mg. substance: 12.405 mg. CO_2 and 6.110 mg. H_2O.
$C_5H_{12}O_2$. Calculated. C 57.69, H 11.53.
Found. " 57.04, " 11.52.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 2.60° \times 100}{2 \times 8.00} = + 16.2°.$$

Di-(Phenylurethane) of Dextro-1,2-Dihydroxypentane.—The urethane was prepared in the usual way and recrystallized from dilute alcohol. It melted at 107–110°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 5.78 cc. 0.1 N acid.
$C_{19}H_{22}O_4N_2$. Calculated. N 8.18.
Found. " 8.09.

In absolute alcohol the optical rotation was

$$[\alpha]_D^{20} = \frac{+ 1.50° \times 100}{2 \times 4.53} = + 16.5°.$$

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[7] Neuberg, C., and Kerb, E., *Biochem. Z.*, 1918, xcii, 96. Färber, E., Nord, F. F., and Neuberg, C., *Biochem. Z.*, 1920, cxii, 313.

XUM

Conversion of Optically Active 1,2-Dihydroxypentane into Methylpropyl Carbinol.

Dextro-1-Bromo-2-Hydroxypentane.—Into 14.5 gm. of 1,2-dihydroxypentane ($[\alpha]_D^{22} = +16.2°$) cooled in an ice water bath were passed 15 gm. of dry hydrogen bromide. The reaction mixture was then heated on the steam bath for ½ hour, cooled, ice and chloroform added, and neutralized with solid potassium carbonate. The bromohydrin was extracted with chloroform and the extract dried over sodium sulfate. The chloroform was removed under reduced pressure and the bromohydrin distilled. It boiled at 72–74°, p = 18 mm. It analyzed as follows:

0.1220 gm. substance: 0.1343 gm. AgBr.

$C_5H_{11}OBr$.	Calculated.	Br. 47.90.
	Found.	" 46.84.

In a 2 dm. tube without solvent it had the following rotation: $\alpha_D^{20} = +10.5°$.

Dextro-Methylpropyl Carbinol.—The bromohydrin obtained in the above experiment was reduced in alkaline solution with hydrogen in the presence of colloidal palladium. The procedure was the same as that previously described for the reduction of 1-iodo-3-hydroxybutane.[8] The ether extract was dried over sodium sulfate and the ether removed with the aid of a Vigreux column. The carbinol was then distilled and a fraction which distilled at 116–120° was collected. In alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 2.00° \times 100}{2 \times 7.00} = + 14.3°.$$

α-Naphthylurethane of Dextro-Methylpropyl Carbinol.[9]—The urethane was prepared in the usual way. Recrystallized from dilute alcohol, it melted at 94–96°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 4.13 cc. 0.1 N acid.

$C_{16}H_{19}O_2N$.	Calculated.	N 5.44.
	Found.	" 5.78.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 1.05° \times 100}{2 \times 2.97} = + 17.7°.$$

[8] Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxi, 467.

[9] Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 505.

THE EFFECT OF ANESTHESIA ON THE RECOVERY PROCESS IN MAMMALIAN SKELETAL MUSCLES.

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INTRODUCTION.

The work of Fletcher and Hopkins (1), Meyerhof (2), Embden (3), Hill (4), and others seems to have established beyond doubt that in the contraction of all muscles[1] a definite cycle of changes occurs in the carbohydrate metabolism. Briefly, glycogen is

[1] While it has yet to be definitely established that the contraction of the heart is dependent on a glycogen-lactic acid cycle such as occurs in skeletal muscle, there is no doubt that this is so in the case of smooth muscle (Evans (5)). A few experiments of my own on dog heart seem to show that glycogen disappearance during rigor mortis is accompanied by an equivalent appearance of lactic acid. It will be surprising if the contraction of the heart is dependent on some other chemical mechanism than this.

broken down to lactic acid *via* a hexose phosphate (lactacidogen) and contraction occurs. This is the anaerobic phase. During recovery one-fifth to one-sixth of the formed lactic acid is burnt and the remainder reconverted into glycogen—by what route there is, at present, a difference of opinion. This is the aerobic phase and will not occur at all if oxygen is absent.

The initial purpose of this investigation was to try to get some idea of the time taken in the intact mammal (cat) for this resynthesis of glycogen during recovery to be completed.

Hill, Long, and Lupton (6) found in man, after exercise, that the greater part of recovery occurred within ½ to ¾ of an hour, although if the exercise were severe, recovery was not completed for over 3 to 4 hours. In these experiments the excess oxygen usage after exercise was taken as a measure of the lactic acid burnt, and hence its coincident resynthesis was assumed to have approximately the same time relations.

The point that arises is this: When recovery is complete, as judged by the return of the oxygen consumption to normal, has the glycogen content of the muscles actually performing the exercise returned to what it was before exercise, or has a large quantity of the lactic acid formed in them been removed by the liver and those muscles not participating in the effort, so that while recovery of the whole animal is complete the active muscles have still less than their initial content of glycogen? So far this question has not been entirely answered. Barr and Himwich (7) have shown that the lactic acid formed in the legs during exercise is removed by the passive arm muscles, while Meyerhof (8), Embden (9), and others, have shown that muscle pulp or isolated muscles can convert lactic acid to glycogen in the presence of oxygen.

It was thought that it could be easily done by stimulating one of a pair of corresponding muscles of anesthetized cats for a definite time, and by a series of experiments determining how long it took for the glycogen contents of the stimulated one to return again to the same value as the control.

As will subsequently be shown, the recovery processes are profoundly altered by the presence of the anesthetic. The effect seems to be a blockage of the resynthesis of the formed lactic acid; that is, of the oxidative phase. The initial breakdown of glycogen and the initiation of contraction do not appear to be affected.

Methods.

Cats were the animals used in all the experiments, nearly a hundred of which have been performed. In some a general anesthetic, *i.e.* amytal, was used; in others a local anesthetic only. In a further series decerebrate animals were employed. Muscle glycogen was estimated by Pflüger's method (10), while the sugar formed in its hydrolysis was estimated by Bertrand's method. Muscle lactic acid was estimated by the technique previously described by Katz and Long, this being essentially that of Meyerhof (2) with a few modifications. Blood sugar determinations were made by the Folin-Wu method and blood lactic acid by Clausen's method (11), observing the precautions previously noted by myself (12).

Estimation of Glycogen and Lactic Acid in Mammalian Muscles and Heart.

The tissues (10 to 20 gm. are sufficient) are removed as rapidly as possible and, in the case of the heart, are squeezed free from blood, and weighed. They are then finely chopped in alcohol at −6° or less, care being taken that this is done beneath the surface of the liquid. The whole mixture is then left in the freezing mixture for ½ hour and preferably longer.

For the estimation of lactic acid and glycogen in the same tissue it is ground in a mortar with fine quartz sand and sufficient 95 per cent alcohol to make a paste, the alcohol being run off and replaced by fresh about half a dozen times. The mixture of sand and tissue is then placed on a filter paper and alcohol run through. The filtrate is then poured back through the filter, this being repeated some half a dozen times. Finally a quantity of fresh alcohol is run through. This procedure will extract the greater part of the lactic acid while not affecting the glycogen which is insoluble in 95 per cent alcohol. The alcoholic extract is then worked up for lactic acid as described later.

To estimate glycogen in the fresh tissue or in the residue left after the alcoholic extraction, 2 cc. of 40 to 60 per cent KOH are added for every gm. of tissue used. The mixture is heated in a water bath for 3 hours or better still, in an autoclave at 120° for 2½ hours. It is then cooled, diluted with twice its volume of

distilled water and the glycogen precipitated with 4 times its original volume of 95 per cent alcohol. It is then left to stand overnight, by which time the glycogen will have settled in a fairly compact layer at the bottom of the beaker. The supernatant fluid is decanted off through an asbestos filter. If this is done carefully no glycogen will go into the filter; if it does, the filter pad must be added to the precipitate. The whole of the remaining fluid and precipitate is evaporated to dryness on the water bath, is dissolved in a small quantity (10 to 20 cc.) of water, and 2 to 3 drops of phenolphthalein added and the solution neutralized with 10 per cent HCl. Double the bulk of 95 per cent alcohol is added and the mixture is again left overnight to settle. Next day the supernatant fluid is again decanted off, the remainder evaporated to dryness, and redissolved in water. 5 cc. of 5 per cent HCl are then added and the mixture boiled gently for 3 hours to hydrolyze the glycogen. The solution is cooled, neutralized, filtered if necessary, and either diluted to an appropriate volume or else used for a single estimation of the glucose. In the latter case the volume should be approximately 20 cc.

The glucose is estimated by Bertrand's method which is too well known to need detailed description. If very small amounts of glycogen are present, or if the original amount of tissue is small, it is necessary to estimate the glucose by a micro method (Folin-Wu or Hagedorn-Jensen).

The estimation of lactic acid[2] in the alcoholic extract is done by Meyerhof's method (2). The solution is evaporated to dryness and the residue extracted with successive small quantities of a saturated solution of ammonium sulfate. For the above quantities of tissue a total quantity of 20 cc. is sufficient. The extract is filtered through asbestos and extracted three times with successive 10 cc. portions of pure benzene. This removes traces of alcohol, *etc.* The solution is then heated on a water bath to remove traces of benzene and is diluted with distilled water to 100 cc. 10 cc. of this are taken and the lactic acid estimated by the permanganate oxidation method of Clausen (11).

[2] The lactic acid method described above does not give absolute values as the alcohol extracts other substances besides lactic acid that reduce permanganate. To get a true value further purification is necessary. Nevertheless it gives fairly accurate relative values, as the other alcohol-extractable constituents of muscle are not affected by stimulation.

The routine of a typical experiment under general anesthesia was as follows: The animal was anesthetized, usually with amytal in doses of 0.5 to 0.7 cc. of a 10 per cent solution of the sodium salt which was injected intraperitoneally. Both sciatic nerves were then cut, high up in the thigh, and one was placed on platinum electrodes. Cannulæ were tied in the trachea and the carotid artery—a blood sample being withdrawn as a resting control for blood sugar and blood lactic acid. One sciatic nerve was then stimulated by maximum break shocks from a Lewis rotary contact at a rate of 60 per minute for 30 minutes. This rate and time of stimulation were used in the majority of experiments. Furthermore the rate of stimulation is not rapid or severe enough to send the muscles into great "oxygen debt." That this is so, is shown by the fact that no great increase of lactic acid occurred in the blood, and furthermore, the strength of response to the stimulation remained approximately constant throughout the experiment. In other words, the muscles were in a "steady state."

When the stimulation was stopped, the gastrocnemius muscles were either removed at once or the wounds were closed and the animal was left to recover, for a period varying from ½ to 7 hours. In some cases the operation was performed as aseptically as possible and the animal allowed to recover from the anesthetic before the muscles were removed. In all cases on removal the muscles were rapidly weighed and chopped at once in cold 95 per cent alcohol to prevent postmortem breakdown of glycogen. When recovery was allowed two or three additional blood samples were taken—one always at the end of the period of stimulation.

The experiments under local anesthesia were performed by holding the cat firmly but gently on its back, so as to prevent any struggling. The skin areas over the exit of the great sciatic nerve from the pelvis were carefully anesthetized with 1 per cent novocaine solution. The sciatic nerves were then dissected down to, and lifted out and severed by one snip of the scissors. Usually this invoked but one response from the muscles supplied by it.

The distal end of one nerve was placed on the electrodes and from here on the technique was the same as previously described. The animals were killed by the injection of massive doses of amytal intraperitoneally. This rapidly produces unconsciousness without struggling, upon which the tissues were removed.

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In the decerebrate experiments the animals were anesthetized with ether and the carotid arteries tied. The skull was then trephined and the brain stem sectioned at the required level with a fine blunt-ended piece of glass tubing. Hemorrhage is almost negligible by this method.

Results.

1. Comparative Glycogen Content of the Gastrocnemius Muscles of the Cat.

The first essential of these experiments is that the glycogen content of the two gastrocnemius muscles should be equal, or at

TABLE I.

Glycogen Content of Right and Left Gastrocnemius Muscles of the Cat (Control Experiments).

Experiment No.	Right gastrocnemius.	Left gastrocnemius.	Difference.
	gm. per 100 gm.	*gm. per 100 gm.*	*per cent*
3	1.34	1.40	4.3
6	0.87	0.90	3.3
9	1.22	1.13	6.5
10	0.84	0.81	3.6
12	0.71	0.71	0.0
13	0.70	0.70	0.0
15	1.16	1.14	1.8
17	0.59	0.56	5.1
18	0.76	0.82	7.3
19	0.80	0.80	0.0
Mean.....	0.90	0.90	3.2

least sufficiently so to render the experimental results of some value. Elias and Schubert (13) have shown that the glycogen contents of paired mammalian muscles are equal to within 2 to 3 per cent. Best and his coworkers (14) have found similar results in the cat and quote Pflüger (15) as supporting evidence.

Table I shows my own results on a series of ten cats in which the mean difference of this series was 3.2 per cent, while the individual variations were from 0.0 to 7.3 per cent. As will be seen later, the variations recorded under the experimental conditions used were much greater than this. The average glycogen content of

the cat gastrocnemius muscle is about 0.9 per cent but is subject to considerable variation from animal to animal.

2. Effect of Anesthesia without Stimulation on Glycogen Content of Skeletal Muscles.

It is a well known fact that exposure of isolated muscles to chloroform vapor brings about a rapid breakdown of the lactic acid precursors so that in a very short time, half an hour or less, maximum amounts of lactic acid appear in the muscles.

It is quite possible that this effect of chloroform may occur to some extent in the intact animal, and its possible relation to heart failure under anesthesia will be considered in a later article.

TABLE II.

Effect of Anesthetics without Stimulation on Glycogen Content of Muscles.

Experiment No.	Anesthetic.	Duration of anesthesia.	Glycogen per 100 gm.		Difference.
			Right gastrocnemius removed at start.	Left gastrocnemius removed at end.	
		hrs.	*gm.*	*gm.*	*per cent*
64	Amytal.	3	0.74	0.59	20.3
65	"	3	0.70	0.58	17.1
79	Ether.	3	0.67	0.55	17.9
Muscles removed together after 3 hours of anesthesia.					
73	Amytal.	3	0.70	0.67	4.3
74	"	3	0.81	0.73	9.9

Meantime, it becomes of some interest to determine the effect of the anesthetics used in these experiments on the resting skeletal muscles. After anesthetizing, one gastrocnemius was removed at once while the other was left *in situ* and exposed to the anesthetic for 3 hours before being removed. The results are shown in Table II and indicate that this exposure has resulted in a 17 to 20 per cent reduction in glycogen content. Best, Hoet, and Marks (14) found a similar effect from amytal. If both muscles are exposed for 3 hours to the anesthetic, the difference is within the experimental one for this method (see Table I).

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TABLE III.

Glycogen Restoration, Amytal Anesthesia.

Stimulation rate, 60 per min. for 30 min.

	Experiment No.	Time of recovery.	Glycogen per 100 gm.		Difference.	Remarks.
			Stimulated muscle.	Control muscle.		
			gm.	gm.	per cent	
Series A.	21	No recovery.	0.34	0.89	62.0	
	22	" "	0.37	0.92	60.0	
	23	" "	0.40	0.76	47.4	
	24	" "	0.48	1.10	56.4	
	25	" "	0.22	0.97	75.5	Strychnine convulsions.
	30	" "	0.28	0.59	52.6	
	37	" "	0.30	1.00	70.0	
	40	" "	0.43	1.02	57.8	
	53	" "	0.53	1.12	52.5	Mean excluding Experiment 25, 57.3.
Series B.	32	½ hr.	0.33	0.95	65.4	
	33	1 "	0.44	0.88	50.0	
	34	2 hrs.	0.39	0.89	56.2	
	36	3 "	0.36	0.92	60.9	
	51	3 "	0.56	1.01	44.5	
	54	3 "	1.06	1.71	39.0	Light anesthesia.
	59	3 "	0.22	0.52	57.6	
	69	3 "	0.30	0.63	52.4	
	72	3 "	0.39	0.61	36.0	
	108	3 "	0.42	0.68	38.3	Mean of Experiments 32 to 108, 50.3.
	39	5 hrs.	0.35	0.67	47.8	
	26	7 "	0.74	0.97	23.7	
	42	7 "	0.71	1.06	33.0	
	28	7 "	0.95	1.01	5.9	Strychnine convulsions. Very light anesthesia.
	66	24 "	0.59	0.75	21.2	Anesthetized throughout.
	68	24 " *	0.55	0.52	5.5	After 24 hrs. recovery from anesthesia.
	71	5 days.*	0.60	0.63	4.8	After 5 days recovery from anesthesia.

* *Cf.* text.

3. Effect of Stimulation on Glycogen Content of Skeletal Muscles.

(*a*) *With General Anesthesia.*—In the great majority of these experiments the anesthetic used was amytal, made by Eli Lilly and Company. This is isoamylethylbarbituric acid. In the first series of experiments (Table III, Series A) the muscles were taken immediately at the end of stimulation of one of them. This stimulated side showed an average reduction of 57.3 per cent in glycogen content compared to the resting side. Even at first sight this seems a large reduction considering that the stimulation was only at the rate of 60 a minute for 30 minutes, and that no great increase could be observed in muscle lactic acid or blood lactic acid as a result of it.

In Series B, Table III, the effect of periods of recovery from ½ to 24 hours was observed. During this recovery period the animal was left anesthetized. A study of Table III shows that recovery after 3 hours had entirely unaffected the difference in glycogen content of the two sides, the average difference of the six experiments allowing 3 hours recovery, being 50.3 per cent, as compared to 57.3 per cent when the muscles were removed at the end of stimulation.

Recovery for longer than 3 hours seems to show that a certain restoration of glycogen is taking place in the stimulated muscles, the average difference being about 25 per cent at 7 hours, while if a further injection of amytal was given (so as to maintain the anesthesia over until next day) the difference was still 22 per cent. In two other experiments the muscles were not removed until the animals had recovered from the anesthetic for 24 hours and 5 days respectively, at which time the difference in glycogen content was only 5.5 and 4.8 per cent respectively.

The slow restoration of glycogen in these experiments is in sharp contradistinction to the lactic acid changes. The lactic acid content both in these cats and in man (6) after exercise bears an entirely different time relation. In my direct determinations on animals, the muscle lactic acid after exercise of this kind is never greatly increased. In addition, any accumulation is removed within an hour of exercise, although, as previously pointed out, this may be due to its being washed out by the blood stream. In man the recovery process, that is the restoration and oxidation of

lactic acid formed as a result of exercise, is complete even after the severest exercise within 3 to 4 hours (6). Here again it is just possible that this has been done by the inactive muscles and the liver, while the restoration of the glycogen content of the active muscles is a much slower process, extending over at least 24 hours and being dependent upon sugar brought to them by the blood stream. In my opinion, this latter view is unlikely as, if glycogen restoration after such mild exercise as was done by these cat muscles is so slow, why is it that in man several maximum efforts can be made by the same groups of muscles within 2 to 3 hours? (*Cf.* any major athletic event.)

TABLE IV.

Glycogen Restoration, Local Anesthesia.

Stimulation rate, 60 per min. for 30 min.

Experiment No.	Time of recovery.	Glycogen per 100 gm.		Difference.
		Stimulated muscle.	Control muscle.	
	hrs.	*gm.*	*gm.*	*per cent*
74	3	0.69	0.70	1.4
91	3	0.58	0.57	1.7
107	3	0.39	0.44	11.4
110	3	0.76	0.79	3.8
112	3	0.46	0.50	8.0
114	3	0.67	0.70	4.3
Mean				5.1

(*b*) *In Decerebrate Animals.*—On the assumption that this inhibition of glycogen restoration was an effect of the anesthetic, a similar experiment was performed on a decerebrate cat. In this experiment ether was used only as a preliminary and the animal was allowed to breathe the majority of it off before one muscle was stimulated. The result was that the glycogen content of the control and stimulated muscles differed only by 8.5 per cent after 3 hours recovery.

(*c*) *With Local Anesthesia.*—Decerebration may introduce so many factors known and unknown, into muscle metabolism that it was not proceeded with. Instead, both sciatic nerves of a cat were severed under local anesthesia and the experiment proceeded with as be-

fore. In all experiments in Table IV 3 hours recovery were allowed, and as can be seen recovery was practically complete in this time. The remarkable difference in results, as shown in Tables III and IV, can only be put down to the presence of general anesthesia in the former series of experiments.

(*d*) *Changes in Muscle Lactic Acid during General Anesthesia.*—Table V shows the changes in lactic acid content of the muscle in experiments parallel to those in Tables III and IV. In some instances both lactic acid and glycogen were determined in the same pairs of muscles. After killing in ice-cold alcohol, they were ground thoroughly with sand and extracted with further quantities

TABLE V.

Lactic Acid Production in Muscles, Anesthesia.

Stimulation rate, 60 per min. for 30 min.

Experiment No.	Anesthetic.	Time of recovery.	Lactic acid, mg. per cent.	
			Resting muscle.	Stimulated muscle.
43	Amytal.	None.	0.40	0.15
53	"	"	0.07	0.09
56	Decerebrate.	"	0.12	0.22
57	"	"	0.15	0.26
52	Amytal.	3 hrs.	0.10	0.20
54	"	3 "	0.04	0.05
55	Decerebrate.	3 "	0.10	0.10
58	"	3 "	0.14	0.13

of 95 per cent alcohol. The alcoholic filtrates were worked up for lactic acid, while the residue of sand and muscles was treated with 60 per cent KOH and the glycogen determined as before. It will be seen that the lactic acid content immediately after stimulation is never more than twice as great as the resting value, while 3 hours later the two are the same within experimental limits.

(*e*) *Effect of Amytal Anesthesia on Blood Sugar and Blood Lactic Acid after Stimulation.*—Page (16) reports that amytal anesthesia is without effect on the blood sugar. This, from my own experience, is not invariably the case and Weiss (17) reports similar results. After stimulation of the one set of muscles the blood sugar is often slightly raised, but never to any great extent.

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The blood lactic acid did not change very much after stimulation, probably owing to the fact that the amount produced was diluted to too great an extent by the circulation and tissue fluid. A simple calculation will show this:

Cat, weight 3 kilos; assume 1½ kilos in diffusion equilibrium with the blood stream. Glycogen content of stimulated muscles, 0.3 per cent; glycogen content of the resting muscles, 1.0 per cent; weight of muscles

TABLE VI.

Lactic Acid in Blood after Stimulation of Muscles.

Stimulation rate, 60 per min. for 30 min. Amytal anesthesia throughout.

Experiment No.	Duration of recovery.	Lactic acid, mg. per cent.			Remarks.
		Resting sample.	End of exercise.	End of recovery.	
30	None.	15.4	15.0		
24	"	20.3	230.0		Strychnine spasms for 20 min.
25	"	22.8	280.0		Strychnine spasms for 1 hr.
37	"	33.9	28.1		
41	"	33.2	31.6		
44	"	23.9	34.9		
53	"	20.1	27.5		
32	½ hr.	41.1	40.0	29.0	
33	1 "	48.5	44.1	32.5	
34	2 hrs.	24.1	26.3		
36	3 "	31.8	47.5	33.9	
52	3 "	27.7	27.7	19.1	
54	3 "	38.6	37.2		
59	3 "	34.8	41.7	17.9	
26	7 "	26.7	38.5	17.5	
42	7 "	56.8	68.6	40 7	

stimulated, 40 gm. Therefore, the glycogen loss equals 0.28 gm., which is equivalent to 0.26 gm. of formed lactic acid. Therefore, the percentage increase in the blood and tissue fluid would be $\frac{100 \times 260}{1500} = 1.7$ mg. (approximately), an undetectable amount.

After 3 hours recovery the lactic acid often fell slightly below the resting figure, as this was sometimes upset by movements during the administration of the anesthetic.

4. Oxygen Intake during and after Stimulation under General Anesthesia.

The present accepted view is that the excess oxygen intake during exercise and the excess oxygen used in recovery are required entirely for the restoration of formed lactic acid to its precursor and ultimately to glycogen. Whether this is accomplished by the oxidation of one-fifth to one-sixth of the formed lactic acid, as is postulated by Hill (4) and Meyerhof (2) or whether through other metabolic paths, is not yet definitely decided. Nevertheless, if anesthesia abolishes this restorative or oxidative phase we would expect to find that this excess usage of oxygen was absent.

I attempted to do this in a number of animals by attaching respiratory valves made of sausage skin to the tracheal tube and collecting the expired gases in small bags after the Douglas bag pattern. Owing to the comparatively small weight of muscle stimulated, it is not to be expected that the excess oxygen usage would be great, and as a result these experiments have up to the present been negative. They are, however, being repeated in animals convulsed by strychnine. The technique employed is essentially the same as used by Hill, Long, and Lupton (6) in their experiments on the oxygen requirement of exercise in man.

5. Fate of Injected Sodium Lactate in Anesthetized and Unanesthetized Animals.

From the generally accepted views of muscular contraction it is to be expected that injected sodium lactate would be partially oxidized and partially converted into glycogen. If the oxidative phase is blocked by the presence of anesthetics, injected sodium lactate would first come into equilibrium with the tissue fluids and then its level in the blood and tissue fluids should remain constant until the anesthetic is removed.

Riegel (18) has studied the fate of injected sodium lactate in unanesthetized dogs, while Katz and I in some unpublished experiments have done the same thing on anesthetized ones. The results will be considered in full in a later paper, but, for the present, it is sufficient to say that in our experiments and Riegel's the rate of removal from the blood after the first rapid fall due to diffusion is

either nil or very slow. This is especially seen when the rate of removal in these experiments is contrasted with the rate of removal of formed lactic acid in man (6).

The presense of an anesthetic in our experiments apparently does not cause any different rate of removal from that found by Riegel in unanesthetized animals. Yet both are much slower than is the removal of lactic acid formed in the body as the result of exercise. Why is there this difference?

At present the only explanation that I can offer is that we are dealing in the experiments of Riegel and of Katz and myself with the racemic (inactive) form of lactic acid, while the lactic acid produced in the muscles is the dextrorotatory type. The rate of oxidation of these isomers may be very different and account for these results.

There are many instances of this difference between optical isomers in their power to produce physiological effects. To quote but two: (1) the difference in pressor effect of the two optical isomers of adrenalin, the levo being 11 to 13 times as active as the dextro; (2) *d*-glucose is probably the only form oxidized in the body, *l*-glucose being almost quantitatively excreted unchanged. The close connection between glucose and lactic acid is to be remembered in view of the above, since the greater part of carbohydrate oxidation occurs in the muscles.

However, this question is still in the experimental stage and I hope to deal further with it in a later communication.

DISCUSSION.

So far the definite finding on the effect of anesthesia in the recovery process in muscle has been (1) an almost complete inhibition of the restoration of glycogen in the muscles; (2) the contraction phase of the glycogen lactic acid cycle is unaffected. The lactic acid formed in contraction disappears from the muscles and probably comes into the diffusion equilibrium with the body fluids. No doubt a certain amount finds its way into the urine in spite of the scantly kidney secretion that is going on under anesthesia. Although not yet definitely proved, it would appear from the above consideration that the usual response of the oxygen intake to exercise would be greatly diminished or lacking under anesthesia.

Lack of knowledge of the fundamental oxidative mechanism of the cell prevents us from attempting to state the exact reason why anesthetics should act in this manner on the carbohydrate cycle in muscles. No doubt this is a highly specialized mechanism and as such is readily susceptible to the effect of anesthetics (*cf.* the central nervous system). The work of Warburg (19) on the effect of hypnotics of the veronal type (of which amytal is one) on cell oxidation is particularly suggestive in this respect.

Chloroform is well known to have a remarkable effect in breaking down the lactic acid precursors in muscles exposed to its vapor outside the body. As far as I know, no one has attempted to test the power of other anesthetics in this respect, though if they act in a similar manner it seems probable that another factor might be introduced into the effects of them on muscle metabolism even in the low concentration in which they are present in the blood. Two effects would then be at work: (*a*) an inhibition of the oxidative-restorative phase and (*b*) a direct breakdown of lactic acid precursors apart from that caused by the stimulation of the muscles due to the specific effect of the anesthetic used.

The practical applications of these experiments concern only those muscles which are essential to life during anesthesia; *i.e.*, the heart and respiratory muscles. Dr. Brow and I are investigating the changes in the glycogen content of these muscles during anesthesia and the results on the heart (reported in detail in the next part of this work) are the most striking. So far we have found the glycogen content to be greatly lowered by some 3 hours of ether anesthesia and by even shorter times under chloroform. In addition this lowering of glycogen appears to be connected with definite electrocardiographic changes.

The liver glycogen is also lowered by anesthesia, although surgical trauma may account for a certain proportion of this. Nevertheless, the hyperglycemia of anesthesia is a well recognized fact. Meyerhof (2) and Embden (3) have taught us how closely the phosphorus metabolism of the body is bound up with that of the carbohydrates, particularly in the muscles. Stehle and Bourne (20) have shown that during anesthesia there is a retention of inorganic phosphates in the body and that these are not removed until the anesthetic is withdrawn. Now the immediate precursor of lactic acid according to Meyerhof (2) is a hexose diphosphate

which on breakdown gives lactic and phosphoric acid by a mechanism such as this.

$$1.\ \frac{5}{n}\ \underset{\text{(glycogen)}}{(C_6H_{10}O_5)n} + 5\ H_2O + 8\ K_2HPO_4$$

$$= 4\ C_6H_{10}O_4(PO_4K_2)_2 + C_6H_{12}O_6 + 8\ H_2O$$

(hexose diphosphate)

$$= 8\ \underset{\text{(lactic acid)}}{C_3H_6O_3} + 8\ \underset{\text{(dipotassium hydrogen phosphate)}}{K_2HPO_4} + C_6H_{12}O_6$$

$$2.\ 8\ C_3H_6O_3 + 8\ K_2HPO_4 + C_6H_{12}O_6 + 6\ O_2$$

$$= 4\ C_6H_{10}O_4(PO_4K_2)_2 + 6\ CO_2 + 14\ H_2O$$

$$= \frac{4}{n}\ (C_6H_{10}O_5)n + 8\ K_2HPO_4 + 6\ CO_2 + 10\ H_2O$$

If the oxidative phase is inhibited by anesthesia, it is to be expected that inorganic phosphates would accumulate just as the lactic acid does. Furthermore, a direct action of anesthetics in breaking down the lactic acid precursors would have a similar effect as regards the phosphates. As Stehle and Bourne (20) have suggested, this effect must play no small part in the production of the acidosis of anesthesia apart from any incident on the anoxemia accidentally produced by administration of the anesthetic, although this was avoided in all the experiments recorded above.

BIBLIOGRAPHY.

1. Fletcher, W., and Hopkins, G., *Proc. Roy. Soc. London, Series B*, 1907, xxxv, 247.
2. Meyerhof, O., *Arch. ges. Physiol.*, 1920, clxxxii, 284, and numerous other papers.
3. Embden, G., Schmidt, E., and Wittenberg, M., *Z. physiol. Chem.*, 1913, lxxxviii, 210.
4. Hill, A. V., Muscular activity, Herter lectures for 1924, Baltimore, 1926.
5. Evans, C. L., *Biochem. J.*, 1925, xix, 1115.
6. Hill, A. V., Long, C. N. H., and Lupton, H., *Proc. Roy. Soc. London, Series B*, 1924, xcvi, 438; 1924–25, xcvii, 84, 155.
7. Barr, D. P., and Himwich, H. E., *J. Biol. Chem.*, 1923, lv, 525.

8. Meyerhof, O., Lohmann, K., and Meier, R., *Biochem. Z.*, 1925, clvii, 459.
9. Embden, G., and Laquer, F., *Z. physiol. Chem.*, 1921, cxiii, 1.
10. Pflüger, E., *Arch. ges. Physiol.*, 1909, cxxix, 362.
11. Clausen, S. W., *J. Biol. Chem.*, 1922, lii, 263.
12. Long, C. N. H., *Proc. Roy. Soc. London, Series B*, 1924, xcvi, 444.
13. Elias, H., and Schubert, E., *Biochem. Z.*, 1918, xc, 229.
14. Best, C. H., Hoet, J. P., and Marks, H. P., *Proc. Roy. Soc. London, Series B*, 1926–1927, c, 32.
15. Pflüger, E., Glycogene, in Richet, C., Dictionnaire de physiologie, Paris, 1906, vii.
16. Page, I. H., *J. Lab. and Clin. Med.*, 1923–24, ix, 194.
17. Weiss, S., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 363.
18. Riegel, C., *J. Biol. Chem.*, 1927, lxxiv, 135.
19. Warburg, O., *Biochem. Z.*, 1921, cxix, 134.
20. Stehle, R. L., and Bourne, W., *J. Biol. Chem.*, 1924, lx, 17.

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QUANTITATIVE STUDIES OF β-OXIDATION.

I. THE CONJUGATION OF BENZOIC ACID AND PHENYLACETIC ACID FORMED AS THE END-PRODUCTS FROM THE OXIDATION OF PHENYL-SUBSTITUTED ALIPHATIC ACIDS.*

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As the result of Knoop's studies on the oxidation of the simple phenyl-substituted normal aliphatic acids, he proposed his well known theory of β-oxidation (1) about which is centered so much of our present knowledge concerning the metabolism of fats. The intensive researches of Dakin (2–5) on the oxidation of these same acids especially from the point of view of isolating the intermediary products further substantiated this hypothesis and presented evidence to explain the mechanism involved in this important biological oxidation. Various studies on the formation of the acetone bodies have furnished additional proof as to the validity of assuming that β-oxidation is an important mechanism in the combustion of at least the short chain fatty acids. Nevertheless, the question remains whether oxidation through the β-carbon atom is the sole process by which the fatty acid molecule is broken down, or whether other mechanisms such as fission at points of unsaturation also take place. There is no quantitative evidence at present to prove that β-oxidation is the only process employed in the catabolism of fatty acids. Recently Clutterbuck and Raper (6) even suggested that γ- and δ-oxidation may occur quite commonly within the organism. It must be pointed out that the studies of both Knoop and Dakin were essentially qualitative in nature, and that in the few experiments in which yields of hippuric acid and other excretory end-products were given, they

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fall far below the theoretical amounts which should have been obtained from the respective phenyl derivative of the fatty acid under investigation.

The situation has been admirably summed up by Leathes and Raper (7).

> "One gramme molecule of phenylpropionic acid, for instance, should give rise to 1 gramme molecule of benzoic acid and 1 of phenylbutyric acid to 1 of phenylacetic acid. The extent to which these expectations are realized may, therefore, be used as a measure of the completeness of β oxidation. It is unfortunate that the experimental data available which can serve as evidence for the discussion of this question is very scanty..... [After citing several experiments:] So far as these examples go, they do not support the idea of exclusive β oxidation."

When one reviews the experiments of Knoop and of Dakin in the light of our present knowledge concerning the conjugation of benzoic acid in the dog and other animals, the reason for their failure to get quantitative results is obvious. Their attention was centered entirely on the glycine derivatives of benzoic acid and phenylacetic acid, without giving any consideration to other possible conjugated forms in which these acids might be excreted. Since in dogs, the animal used in many of their experiments, benzoic acid is conjugated to the extent of 70 per cent with glycuronic acid (8) it is obvious why the quantity of hippuric acid isolated fell far below the theoretical expected amount. As has been mentioned in a previous paper (9) phenylacetic acid is likewise excreted partly combined with glycuronic acid and partly with glycine. And curiously enough while the ratio of benzoic acid combined with glycuronic acid to that combined with glycine is roughly 3:1, almost the inverse ratio holds for phenylacetic acid, in which case only 1 molecule of the acid is combined with glycuronic acid for every 2 united with glycine. These ratios are so strikingly different, and yet so remarkably constant that they immediately suggest investigating the conjugation of these two acids when they are formed as the end-products of the higher acids of the series. It was this phase of the problem which yielded the most interesting results and was therefore most carefully studied.

EXPERIMENTAL.

The dogs were kept on a standard diet of lard, sucrose, casein, and brewers' yeast as described in a previous publication (8).

TABLE I.

Relative Conjugation with Glycuronic Acid and Glycine of Benzoic Acid and Phenylacetic Acid When Fed or Derived from Oxidation of a Higher Phenyl-Substituted Aliphatic Acid.

Dog No.	Substance fed.	Amount.	Equivalent in terms of benzoic acid or phenylacetic acid.	Period of urine collection.*	Combined benzoic acid.				
					With glycuronic acid.		With glycine.		Total.
		gm.	gm.	hrs.	gm.	per cent	gm.	per cent	gm.
7	Benzoic acid.....	5.0	5.0	32	3.65	71.5	1.45	28.5	5.10
8	" "	5.0	5.0	32	3.96	71.5	1.57	28.5	5.53
2	Phenylpropionic acid.	5.0	4.1	48	2.94	75.0	0.98	25.0	3.92
7	" "	5.0	4.1	48	2.77	65.0	1.50	35.0	4.27
8	" "	6.0	4.9	48	3.50	68.0	1.65	32.0	5.15
8	" "	7.4	6.0	32	4.80	73.0	1.80	27.0	6.60
7	Cinnamic acid.....	6.0	5.0	48	3.50	65.0	1.92	35.0	5.42
8	" "	6.0	5.0	32	3.50	66.5	1.75	33.5	5.25
					Combined phenylacetic acid.				
6	Phenylacetic acid....	5.0	5.0	30	1.58	35.5	2.62	64.5	4.20
6	" " ...	5.0†	5.0	48	0.77	19.0	3.30	81.0	4.07
2	" " ...	5.0	5.0	48	1.25	35.0	2.33	65.0	3.58
2	" " ...	5.0‡	5.0	36	0.80	17.5	3.72	82.5	4.52
7	" " ...	5.0†	5.0	36	1.08	20.0	4.38	80.0	5.46
7	" " ...	5.0	5.0	32	1.61	30.5	3.65	69.5	5.26
8	" " ...	5.5	5.5	48	1.74	32.0	3.75	68.0	5.49

* The period of collection was only approximate since the dogs were not catheterized.

† 20 gm. of gelatin included in regular diet.

‡ 5 gm. of glycine included in regular diet.

The animals were fed once a day, and the acid in the form of its sodium salt was incorporated directly in the food. Cinnamic acid was fed as the ammonium salt. In the earlier experiments

(Table I) the animals were not catheterized, but approximate 30 to 48 hour specimens of urine were collected. In order to get more accurate and consistant results it was found necessary to collect urine for definite periods by means of catheterization. Generally a 24 hour and a following 12 hour specimen were collected. The substance under investigation was fed only every 3rd or 4th day to insure complete removal of its end-products before a new drug was fed.

Analytical Methods.

The same methods as outlined in a former paper (8) were used without modification except that it was found advisable to prolong the hydrolysis of hippuric acid with concentrated hydrochloric acid to 1 hour instead of 30 minutes. The author's method for hippuric acid can be applied without any changes to the quantitative determination of phenaceturic acid, and similarly the analytical procedure for glycuronic acid monobenzoate can be used directly for glycuronic acid combined with phenylacetic acid. This compound is undoubtedly glycuronic acid monophenylacetate. Like the benzoic acid compound, it appears to be levorotatory, but becomes dextrorotatory in alkaline solution.

Free benzoic acid and phenylacetic acid were determined only occasionally and the amount was always found to be very small.

Preparation of Phenylbutyric Acid.—The method used was essentially the same as that employed by Dakin (3), but it was found possible to reduce phenylisocrotonic acid with hydriodic acid directly instead of first converting into phenylbutyrolactone, thus eliminating a rather tedious procedure. The details of the method used were as follows: For the preparation of phenylparaconic acid, 100 gm. of anhydrous sodium succinate, 56 cc. of benzaldehyde, and 60 cc. of acetic anhydride were mixed in a Kjeldahl flask, and heated on a water bath for 15 hours (10) or 6 hours at 108–110° (11). The resulting black mass was dissolved in 1 liter of hot water, steam-distilled to remove the excess benzaldehyde, boiled with decolorizing charcoal, and finally acidified. On cooling a crystalline mass consisting mainly of phenylparaconic acid and a little phenylisocrotonic acid separated out. Without further purification, the dried product was distilled under reduced pressure. In this process carbon dioxide split off and the phenyl-

paraconic acid was converted to phenylisocrotonic acid ($C_6H_5CH=CH\ CH_2COOH$) which distilled as a yellow oil that crystallized on standing. This product also contains a little phenylbutyrolactone. To remove this, the crystalline mass was dissolved in an excess of sodium hydroxide solution which was extracted with ether to take out the lactone. On reacidifying the aqueous solution, phenylisocrotonic acid precipitated out in crystalline form. Instead of converting the acid to phenylbutyrolactone as has been done heretofore, it was reduced directly with hydriodic acid. 1 part of the compound was refluxed for 10 hours with 10 parts of hydriodic acid and 1 part of red phosphorus. The solution was diluted with water, cooled, and extracted with ether. In this procedure a little iodine is liberated and will go into the ether solution. It is very essential to remove the free iodine as it interferes with the isolation of crystalline phenylbutyric acid. The ether solution was therefore shaken with a dilute solution of sodium thiosulfate, filtered through a dry filter, and the ether finally distilled off. The phenylbutyric acid was left as a thick oil which on standing crystallized. The yield based on phenylisocrotonic acid was 90 per cent. The acid used in feeding experiments was not further purified, but the acid can readily be obtained in a high state of purity by recrystallization from hot water.

DISCUSSION.

The main aim of this work was to investigate quantitatively the oxidation and conjugation of the various phenyl-substituted fatty acids previously studied by Knoop and by Dakin. As has been stated before, their work on these acids was primarily qualitative, and this has been one of the chief sources of weakness in the theory of β-oxidation. Thus, the isolation of hippuric acids from urine after feeding phenylpropionic acid does not necessarily prove that the acid is completely oxidized to benzoic acid, for even with a quantitative recovery of all the hippuric acid, two-thirds of the ingested acid will be left unaccounted for, and therefore offers no evidence against the assumption, for instance, that the greater portion of the acid may be completely oxidized by some other mechanism. With the recognition that benzoic acid is conjugated both with glycine and glycuronic acid, and since it has further been shown by the author that the sum of the two forms

of combined benzoic acid fairly quantitatively accounts for the amount of acid ingested (8) it was felt that it might be profitable to reinvestigate the oxidation of some of the phenyl-substituted fatty acids especially from the point of view of studying quantitatively the conjugation of the end-products of these acids. Such a study it was felt might aid materially in deciding whether β-oxidation was the sole mechanism by which the fatty acid is broken down in the body. The results obtained in this research show definitely that the acids thus far studied are broken down exclusively by β-oxidation.

Before discussing the results in detail it might be well to say a little about the experiments themselves. In employing the various phenyl-substituted acids, the idea, of course, is entertained that they are handled by the organism almost exactly like the normal fatty acids until they are oxidized either to benzoic acid or phenylacetic acid. With this in mind all efforts were made in these experiments to preserve as nearly as possible normal physiological conditions. The compounds under investigation were incorporated in the food so that they might enter the body through the same portals and follow the same path as the fatty acids. Parenteral administration was avoided because the rate of absorption is apt to vary greatly and there is a definite danger that one may induce toxic symptoms. To obviate as much as possible any toxic action, the acid studied was administered in relatively small amounts, the equivalent of 0.5 gm. or less of benzoic acid per kilo of body weight. No deleterious effects either immediate or after prolonged feeding were ever noticed. It must furthermore be remembered that small amounts of benzoic acid and possibly also of phenylacetic acid appear so frequently in normal urine thay they can be looked upon almost as constant products of metabolism. This leads one to another point, namely the importance of diets in experiments such as reported in this paper. Obviously, no food containing precursors of benzoic acid ought to be used, nor should the protein fed contain glycine, since this definitely increases the output of hippuric acid. A diet consisting of casein, sucrose, lard, and a little brewers' yeast gave satisfactory results, but nevertheless an appreciable blank for both glycine and glycuronic acid was obtained. The origin of this benzoic acid is not definitely known. Salkowski (12) found

hippuric acid even in the urine of fasted dogs in amounts similar to that obtained in the present study. If the benzoic acid normally found in the urine results from putrefactive processes in the intestines, as is generally assumed, the incorporation of these phenyl-substituted acids should hinder bacterial action by virtue of their antiseptic action, and therefore reduce the blank. Since there is some uncertainty on this point, and as the blank does not materially alter the results, it was thought best to record all experiments without correcting for the blank. By collecting exact 24 hour specimens of urine, one can minimize this small theoretical error, and since it is presumably the same in all experiments, correct comparative results are obtainable. Of course, it is hardly necessary to state that the type of experiment employed in this research does not lend itself for absolute quantitative studies. Among the sources of error one may cite that a small fraction of the substance fed may escape absorption, or that the excretion of the last portion of the substance may be so slow that it is not feasible to collect it completely. Thus, salicylic acid can be detected in the urine even 72 hours after its administration. In general about 80 per cent or more of the benzoic acid or phenylacetic acid, whether derived from higher acids or fed directly, is excreted during the first 24 hours.

The quantitative recovery of benzoic acid and phenylacetic acid following the feeding of the higher phenylaliphatic acids does not constitute the only evidence for exclusive β-oxidation. A second and a more sensitive test was found in the ratios with which benzoic acid and phenylacetic acid combine with glycuronic acid and glycine. For some reason totally unknown, benzoic acid in the dog is mostly conjugated with glycuronic acid, and only to a small extent with glycine, whereas phenylacetic acid, on the contrary, is combined largely with glycine and only to a minor degree with glycuronic acid. In a carefully standardized experiment these ratios are remarkably constant. On giving benzoic acid in a dose of 0.5 gm. per kilo of body weight to a dog maintained on a glycine-free diet, very close to 3 parts of the acid are conjugated with glycuronic acid, and 1 part with glycine. On the other hand, when a similar amount of phenylacetic acid is fed, 2 parts of it will appear in the form of phenylaceturic acid, and only 1 part as the glycuronic acid compound. What is even more

interesting, the benzoic acid which is formed as the end-product of the oxidation of the higher phenyl-substituted acids having an odd number of carbon atoms, is conjugated almost exactly as when benzoic acid itself is fed; and similarly when phenylacetic acid is

TABLE II.

Conjugation of Benzoic Acid and Phenylacetic Acid When Fed Directly and When Derived from Oxidation of Higher Phenyl-Substituted Fatty Acid.

Dog 9. Weight 12 kilos.

Experiment No.	Substance fed.	Amount.	Equivalent in terms of benzoic acid or phenylacetic acid.	Combined benzoic acid.						
				24 hr. period.					Following 12 hr. period.	
				With glycuronic acid.		With glycine.		Total.	With glycuronic acid.	With glycine.
		gm.	gm.	gm.	per cent	gm.	per cent	gm.	gm.	gm.
1	Benzoic acid	6.0	6.0	4.52	74.0	1.60	26.0	6.12	0.48	0.16
2	" "	6.0	6.0	4.50	74.5	1.54	25.5	6.04		
3	Cinnamic acid	7.3	6.0	3.54	70.5	1.50	29.5	5.04	0.67	0.28
4	Phenylpropionic acid	7.4	6.0	3.88	72.0	1.50	28.0	5.38	0.48	0.14
5	Benzoic acid	5.0	5.0	3.65	66.0	1.90	34.0	5.55		
	Phenylacetic acid	1.0	1.0							
	Blank			0.53		0.19		0.72		
				Combined phenylacetic acid.						
6	Phenylacetic acid	6.5	6.5	1.85	34.5	3.50	65.5	5.35	0.58	0.34
7	" "	6.5	6.5	1.85	33.0	3.75	67.0	5.60		
8	" "	4.0	4.0	1.11	30.0	2.56	70.0	3.67		
9	Phenylisocrotonic acid	7.7*	6.5	0.88	31.5	1.89	68.5	2.77	0.54	0.17
10	" "	5.0	4.2	1.11	31.0	2.46	69.0	3.57		
11	Phenylbutyric acid	5.0	4.2	1.08	33.0	2.22	67.0	3.30	0.44	0.20
12	Phenylacetic acid	5.5	5.5	2.42	41.5	3.43	58.5	5.85		
	Benzoic acid	1.0	1.0							

* Dog vomited, amount of material lost not known.

produced as the end-product, the characteristic 2:1 ratio of phenylacetic acid is obtained. In the compounds studied, these two ratios have been so constant that from this fact alone it can be concluded with a fair degree of certainty that both the 3 and 4

carbon acids undergo exclusive β-oxidation. If even less than 20 per cent of the acid studied was oxidized differently, it would undoubtedly be reflected in the ratio, as is well illustrated by Experiments 5 and 12, Table II. By feeding only 1 gm. of phenylacetic acid with 5 gm.of benzoic acid, there was a definite decrease in the amount of glycuronic acid produced, and a corresponding increase in the conjugation with glycine; and when 1 gm. of benzoic acid was fed with 5.5 gm. of phenylacetic acid one obtained almost the theoretical augmentation in glycuronic acid and the expected decreased conjugation with glycine. These illustrations suggest how the ratios of conjugation may offer a new means for

TABLE III.

Relative Rate of Conjugation of Glycuronic Acid and Glycine with Phenylacetic Acid and Benzoic Acid during Early and Late Period of the 24 Hours after Feeding.

Dog 9. Weight 12 kilos.

Acid fed.	Amount.	Combined phenylacetic acid.				Combined benzoic acid.			
		First 8 hrs.		Last 16 hrs.		First 8 hrs.		Last 16 hrs.	
		With glycuronic acid.	With glycine.	With glycuronic acid.	With glycine.	With glycuronic acid.	With glycine.	With glycuronic acid.	With glycine.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Phenylacetic	6.5	0.92	1.30	0.92	2.50				
Benzoic	6.0					1.84	0.55	2.66	0.99

determining the metabolic path of various compounds. This idea will be brought out more clearly when the oxidation of the various acids used in this research will be discussed.

When one attempts to find an explanation for the marked difference with which benzoic acid and phenylacetic acid are handled by the organism, one realizes how incomplete our knowledge is concerning the various synthetic processes in the body. The lack of an adequate supply of either glycine or glycuronic acid certainly seems to be a minor factor, for the experiments recorded in this work indicate that the organism can furnish both substances in relatively large amounts without any embarrassment. The old theory of Magnus-Levy (13) that the glycuronic

acid conjugation is a second line of defense mechanism certainly does not hold in the case of the dog, since the glycuronic acid conjugation seems to be even slightly greater during the earlier part of the period as compared with the formation of hippuric acid or phenylaceturic acid (Table III). This leads to the rather peculiar question whether all of the conjugation with glycine is primary or whether a portion, at least, may not be formed secondarily from the glycuronic acid compound. As was reported in a previous paper (14) when glycuronic acid monobenzoate is ingested by man, the benzoic acid appears entirely as hippuric acid in the urine. While it is very unlikely that all of the hippuric acid is thus formed, one must keep in mind the possibility that at least a part of the glycine compound may be formed in this manner. Considerable light on these questions might be shed if the site of syntheses of these conjugated products were known. While it is probable that glycuronic acid is produced by the liver, there is no direct proof for this assumption. Much conflicting evidence has accumulated on the subject where hippuric acid is synthesized. As this problem is being investigated at present, a full discussion of this subject will be reserved for a later presentation.

In studying the oxidation of phenylpropionic acid, and cinnamic acid, one is immediately struck by the fact that in both instances the conjugation of the benzoic acid, formed as the end-product, is quantitatively much the same as when benzoic acid itself is fed. It had been known even before Knoop's work that both these acids yield hippuric acid. Mattschersky (15) in 1863 noted the increase of hippuric acid after feeding cinnamic acid to dogs, and in 1882 E. and H. Salkowski (16) made the same observation when phenylpropionic acid was fed. But the fact that the benzoic acid both from cinnamic acid and phenylpropionic acid was combined mostly with glycuronic acid was apparently overlooked by these investigators as well as by the later workers in spite of the ease with which it can be detected because of its strong reducing properties. It is remarkable that the benzoic acid whether derived from cinnamic acid, from phenylpropionic acid, or fed directly, should be combined with glycuronic acid and glycine in such constant proportions. From this, one can draw several conclusions. It appears quite definite that the oxidation of both cinnamic acid and phenylpropionic acid must occur rather

promptly after they enter the body, and that both must be handled very much alike. In fact, there are pretty good indications that cinnamic acid may be an intermediary compound in the oxidation of phenylpropionic acid, for on feeding the latter compound, Dakin (4) actually isolated cinnamoylglycocoll in the urine. Whether the cinnamic acid-glycuronic acid compound is similarly formed is not known, but its isolation would be significant since it would lend strength to the author's theory that the fatty acid at some stage of its catabolism is united with a carbohydrate molecule in very much the same manner as benzoic acid is conjugated with glycuronic acid. It should be mentioned at this point that neither cinnamoylglycocoll nor any of the other intermediary compounds such as acetophenone and phenyl-β-oxypropionic acid which Dakin isolated were observed in this work. No special effort was made either to isolate these compounds or even to produce them. It must be recalled that the experiments of Dakin were far more drastic, for the substance studied was given parenterally and often in relatively large doses so that there was a possibility that the oxidative processes of the organism were overwhelmed, allowing an accumulation of the intermediary products. It is doubtful whether more than mere traces of these compounds are excreted when phenylpropionic acid is given *per os*. In further studies on the 3 carbon acids, the oxidation of the possible intermediary compounds especially the keto and the hydroxy acids should be investigated in the same way as phenylpropionic acid itself was studied, since this may help to decide whether either or both compounds are normally formed in the body.

Phenylbutyric acid when fed undergoes exclusive β-oxidation as is well brought out by Experiment 11, Table II, and the end-product, phenylacetic acid, is conjugated in the same ratio as when this acid itself is fed. Phenylisocrotonic acid ($C_6H_5CH=CH\ CH_2COOH$) is handled by the organism exactly like phenylbutyric acid, and this is of real significance. In spite of the fact that the double bond is between the β- and γ-carbon atoms, the split occurs between the α and β position. This shows the ease with which the organism can shift double bonds, and it is important to note that the oxidation does not necessarily occur at the point of unsaturation. Qualitatively, Dakin (3) also observed

that phenylisocrotonic acid yielded only phenylaceturic acid, and he also noted that the various pentenic acids all yielded benzoic acid (hippuric acid) irrespective of the position of the double bonds. Clutterbuck and Raper (6) may be entirely correct in their opinion that γ- and δ-oxidation may occur in the body but only as far as the production of double bonds in the molecule. There is no proof extant that the normal fatty acid is split at any point except between the α- and β-carbon atoms. The production of double bonds in the fatty acid molecule is easily and constantly effected by the organism, but it is unlikely that the fatty acid is split at these points of unsaturation.

From the data obtained on the fate of the 3 and 4 carbon acids in the body, it can be definitely concluded that they are oxidized exclusively through the β-carbon atom. To what extent these results can be employed to explain the catabolism of normal fatty acids is difficult to state. To be sure, this research adds further evidence for the theory that the fatty acids are broken down exclusively by β-oxidation. Whether the constant ratios obtained for the conjugation of benzoic acid and phenylacetic acid are of fundamental importance remains to be tested by further experiments. Preliminary work on phenylvaleric acid seems to show that the benzoic acid derived from this compound is combined in a different ratio from that obtained for the 3 carbon acids. It is obvious that these results cannot be adequately explained by our present knowledge concerning the syntheses which occur in the body. Nevertheless enough progress has been made so that the idea is no longer tenable that these conjugations are more or less unimportant mechanisms concerned solely with the detoxication powers of the organism. If these synthetic processes are looked upon as normal and common chemical reactions made manifest because the body is applying them to a foreign substance, it is possible to perceive how a study of the conjugation of benzoic acid may help to solve various problems of metabolism which at present seems quite unrelated to hippuric acid and glycuronic acid.

The author wishes to thank Miss Dorothea Lemcke who assisted in this research.

SUMMARY.

1. Benzoic acid when fed or derived from the oxidation of cinnamic acid or phenylpropionic acid is conjugated in the dog with glycuronic acid and glycine in the ratio roughly of 3:1.

2. Phenylacetic acid when fed or originating as the end-product of the oxidation of phenylisocrotonic acid or phenylbutyric acid is combined with glycuronic acid and glycine in the ratio approximately of 1:2.

3. The sum of the two conjugated forms either of benzoic acid or phenylacetic acid, excreted during the first 24 hours, accounts for 80 per cent or more of the ingested acid.

4. The study of the oxidation of the 3 and 4 carbon representatives of the normal phenyl-substituted aliphatic acids furnishes additional evidence for the theory that the fatty acids are broken down in the body exclusively by β-oxidation.

BIBLIOGRAPHY.

1. Knoop, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 150.
2. Dakin, H. D., *J. Biol. Chem.*, 1908, iv, 419.
3. Dakin, H. D., *J. Biol. Chem.*, 1908–09, v, 173.
4. Dakin, H. D., *J. Biol. Chem.*, 1909, vi, 203.
5. Dakin, H. D., *J. Biol. Chem.*, 1909, vi, 221.
6. Clutterbuck, P., and Raper H. S., *Biochem. J.*, 1925, xix, 385.
7. Leathes, J. B., and Raper H. S., The fats, London, 1925, 192.
8. Quick, A. J., *J. Biol. Chem.*, 1926, lxx, 59.
9. Quick, A. J., *J. Biol. Chem.*, 1926, lxx, 397.
10. Fittig, R., and Jayne, H. W., *Ann. Chem.*, 1882, ccxvi, 97.
11. Fittig, R., and Leoni, H., *Ann. Chem.*, 1889, cclvi, 63.
12. Salkowski, E., *Ber. chem. Ges.*, 1878, xi, 500.
13. Magnus-Levy, A., *Biochem. Z.*, 1907, vi, 502.
14. Quick, A. J., *J. Biol. Chem.*, 1926, lxix, 549.
15. Mattschersky, P., *Virchows Arch. path. Anat.*, 1863, xxviii, 538.
16. Salkowski, E., and Salkowski, H., *Z. physiol. Chem.*, 1882–83, vii, 161.

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THE EFFECT OF INSULIN ON PROTEIN METABOLISM.*

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An increased heat production but no rise in the carbohydrate combustion was found in some cases in a study of the action of insulin on the respiratory metabolism of normal dogs under amytal anesthesia (1). This raised the question whether the increased metabolism was entirely at the expense of fat or due in part to a catabolism of protein.

Several investigators have observed an increase in urinary nitrogen following the injection of insulin. Such a result was reported by Nash (2) from a control experiment on a phlorhizinized dog which he has interpreted as an increased nitrogen catabolism induced by the hypoglycemia. In normal fasting dogs Sokhey and Allan (3) found that the rise occurred during the first or second 3 hour period and was obtained at each injection during 13 days of fasting. A greater nitrogen excretion on the days that the hormone was given was reported by Labbé and Théodoresco (4) from dogs maintained in nitrogen equilibrium on a specific diet. In fasting rabbits Hawley and Murlin (5) noted a greater protein metabolism for the first 2 hours following the administration of insulin.

Contrary to the work of the investigators mentioned above, we found in some preliminary experiments that the injection of insulin was not always followed by an increase in urinary nitrogen. As it was thought that the state of nutrition of the animals might have an influence on this reaction, the following study was undertaken. Dogs were subjected to various periods of fasting prior to the administration of the hormone, and the nitrogen excretion was accurately determined from hour to hour.

* A preliminary report appeared in *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 170.

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Methods.

In the earlier experiments amytal anesthesia was used in order to follow the procedure of the calorimeter work on insulin (1). Some variation in urinary nitrogen resulted from the use of amytal. In some dogs there was a marked decrease in nitrogen excretion only during the 1st hour after amytal, in others a slightly lower level persisted for several hours, or there was no appreciable change. Therefore in the later experiments no anesthetic was employed.

PROTOCOL 1.

Dog 124; Experiment 39; weight 13.0 kilos.

End of period.	H_2O ingested.	Urine.		Blood sugar.	Notes.
		Volume per hr.	Total N per hr.		
	cc.	*cc.*	*mg.*	*per cent*	
9.40 a.m.					42 hrs. of fasting; catheterized.
10.40 "	40	5	86		
11.40 "	25	1	109		
12.55 p.m.				0.065	6 cc. blood drawn.
1.05 "	25	6	102		
1.10 "					4 units per kilo insulin subcutaneously.
2.10 "	25	1	127		
3.10 "	0	6	148		Hypersensitive.
3.20 "				0.030	6 cc. blood drawn.
4.10 "	0	1	135		
4.20 "	200				100 gm. sucrose ingested.

The urine was collected in hourly periods by catheter and the bladder thoroughly washed five or six times with a total of about 200 cc. of sterile water. The volume of urine secreted was noted. To prevent a diuresis the amount of drinking water was limited during the hours of observation. Blood was drawn from the superficial leg veins for the Shaffer-Hartmann sugar determinations and the Kjeldahl method was used for total urinary nitrogen.

Protocol 1 illustrates a typical experiment. After three or four preliminary control periods to establish the normal level of nitro-

gen excretion the insulin[1] was injected subcutaneously. Urine was then collected for three or four periods until the onset of definite hypoglycemic symptoms when sucrose was given to restore the animal. To demonstrate the potency of the insulin, normal and postinsulin blood sugars were determined in all experiments with the exception of a few in which the animals exhibited very definite convulsions. The insulin dose was 4 or 5 units per kilo of body weight, sufficient to lower the blood sugar to about 0.03 per cent within 2 hours after its administration.

EXPERIMENTAL.

The validity of an increase or decrease in nitrogen metabolism following the injection of insulin depends primarily upon the accuracy with which the level of nitrogen excretion during the preinsulin control period can be determined. Forty-one experiments have been performed on twelve mongrel female dogs varying in weight between 5.3 and 20 kilos, in thirty-five of which no anesthetic was used. In these thirty-five experiments the control urines were collected for three or four consecutive hourly periods at various times during the day between 8.00 a.m. and 6.00 p.m. The individual figures are given in the tables, followed by the mean and its deviation. The smallest deviation from the mean is only ± 0.2 mg. occurring in two experiments, whereas the greatest is ± 6.6 mg. The average deviation for the thirty-five experiments is ± 2.8 mg. of nitrogen per hour. In addition to these data we have the results from forty-nine experiments on exercising dogs which will be published separately, and in which the preliminary control periods are the same as for the insulin experiments. For the most part the animals lie quietly in the metabolism cage, only occasionally moving about therein. The deviation of the mean varies from ± 0.3 to ± 7.6 mg., with an average for the forty-nine experiments of ± 3.0 mg. of nitrogen. These figures indicate therefore that the average of three preliminary periods is an adequate index within a few mg. of the preinsulin level of nitrogen excretion and serves as a control basis for judging variations.

Typical individual experiments on two of the dogs, Nos. 106 and

[1] We are indebted to Eli Lilly and Company for the insulin used in these experiments.

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165, are given in Table I. In the case of Dog 106, following a short fast of 2 days the nitrogen excretion after the insulin injection in the first experiment rose from a level of 132 mg. per hour to 190 in the 2nd hour; and in the second experiment from 120 to 150 in the 1st hour and to 179 in the 4th hour. With the longer fasting periods of 5 and 7 days, there was no significant increase in nitrogen metabolism. Similar results were found with Dog 165; namely, a greater output of nitrogen after 42 hours of fasting but no change in the rate of excretion in the last two experiments with the possible exception of the 4th hour.

TABLE I.

Effect of Fasting Period on Reaction to Insulin.

Urinary nitrogen in mg. per hr.

Dog No.	Experiment No.	Weight.	Fasting period.	Preliminary hrs.				Hrs. after insulin.				Hrs. after sugar following insulin.		Notes.
				1	2	3	Mean and deviation.	1	2	3	4	1	2	
		kg.	*days*											
106	29	9.0	2	137	131	128	**132** ± 2.0	127	190			152	151	
	36	8.8	2	121	121	117	**120** ± 1.0	150		167	179			
	24	8.5	5	107	119	104	**110** ± 3.5	91	105	118				
	26	7.9	7	119	100	103	**107** ± 4.5	110	97					
165	57	8.3	1.75	116	116	124	**119** ± 2.2	136	150	145				
	53	7.9	3	79	72	69	**73** ± 2.2	57	76	76	92			Exercised during fast.
	46	7.1	12	103	111	108	**107** ± 1.8	113	119	101	132			" "

The data from Dog 120, Table II, confirm those of Table I. The first four experiments in the table all show a definite rise after the hormone, whereas in the last three there was little if any change. As might be expected there is some variation in the length of the fasting period necessary to produce the condition in which no increase in protein metabolism after insulin is encountered. It probably depends upon uncontrolled food factors before the fasting period. For example, in Experiments 23 and 44, in Table II, on Dog 120, the increase is definite on the 4th and 5th days of the

fast, while in Experiments 19 and 25 there is no significant increase on the 4th and 6th days of the fast.

That the greater metabolism of nitrogenous material is not due

TABLE II.

Effect of Fasting Period on Reaction to Insulin.

Dog 120. Urinary nitrogen in mg. per hr.

Experiment No.	Weight.	Fasting period.	Preliminary hrs.				Hrs. after insulin.				Notes.
			1	2	3	Mean and deviation.	1	2	3	4	
	kg.	*days*									
21	6.7	2	140	138	142	**140** ± 0.8	175	135	140		
20	6.5	2	113	111	99	**108** ± 3.4	144	113	112		
23	6.5	4	64	64	77	**68** ± 3.4	116	102	92		
44	6.2	5	76	68	76	**73** ± 2.2	93	90	111		
19	6.25	4	83	74	69	**75** ± 3.0	82	79	83	92	
25	6.35	6	65	65	64	**65** ± 0.2	56	71	64		
50	5.3	15	67	65	66	**66** ± 0.4	63	49	39*	46*	Exercised during fast.

* Possibly some urine lost.

TABLE III.

Effect of Insulin on Dogs under Amytal Anesthesia.

Urinary nitrogen in mg. per hr.

Dog No.	Experiment No.	Weight.	Fasting period.	Preliminary hrs.			Hrs. after insulin.			
				1	2	3	1	2	3	4
		kg.	*days*							
30	7	9.2	1	40	73	75	105	93	107	83
	11	8.4	7	56	71	75	79	78	59	59
	5	9.0			85	63	103	138	130	
186	62	14.9	2		190	209	248	208	193	
35	1	8.8			105	109	110	127	127	125

to the muscular exercise and hypertonicity of the insulin convulsion is seen from Table III. In these experiments the dogs were completely anesthetized with amytal (isoamylethylbarbituric acid).

Experiments 7 and 11 on Dog 30 (Table III) exhibit the same effect of the fasting period as is seen in Tables I and II. In both these experiments because of the amytal the first preliminary hour was low, then a level of between 70 and 75 mg. per hour was reached. After the short fasting period (Experiment 7) the increased nitrogen output is evident, while with 7 days of fasting (Experiment 11) there is none. The other three cases in Table III merely emphasize the fact that this phenomenon occurs under conditions in which muscular movement is prevented.

The above observations are all made on single experiments during separate fasting periods. In a different type of experiment several insulin injections were given at 4 or 5 day intervals during

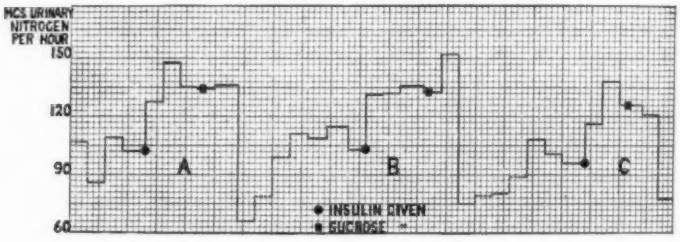

CHART 1. The effect of insulin and of sucrose on nitrogen metabolism during different periods of fasting. The continuous line represents the urinary nitrogen in the day sample, three spaces representing 1 hour. *A*, 2nd day of fasting; *B*, 6th day of nitrogen fasting; *C*, 11th day of nitrogen fasting. The dotted line represents data of the night samples, the length of the periods not being represented.

a period of continuous nitrogen fasting. The results from Dog 124 are illustrated in Chart 1. In each instance sucrose was given 3 or 4 hours after the insulin to relieve the hypoglycemia, thus the animal was nitrogen-fasted for the entire period but carbohydrate-fasted for only 4 or 5 days at a time. With each injection of the hormone, nitrogen excretion was increased, although not to such a great extent on the 11th as on the 2nd or 6th day. The characteristic nitrogen-sparing action of the carbohydrate is seen in the decrease from the preliminary level of 107 mg. per hour on the 2nd day to 66 on the 3rd day and similarly from 111 to 75 on the 6th and 7th days. In a later experiment on Dog I24 in which

no food is taken for 14 days insulin produces no increase in the urinary nitrogen. Similar results were obtained from Dog 123 over a period of 16 days.

DISCUSSION.

The data presented above to the effect that in dogs on short fasting periods the injection of insulin produces a rise in nitrogen output are in agreement with the work of previous investigators. We believe the evidence favors the idea that this increase represents the product of a definite metabolic reaction and not merely a washing out of accumulated inert nitrogenous material. The measurements of the volume of urine secreted show that there is no diuresis accompanying the increase in urinary nitrogen but that on the contrary a very constant volume of urine is secreted throughout the experiments. If sugar is given with the insulin, there is no extra nitrogen excretion according to the work of Sokhey and Allan (3) which is confirmed by one of our experiments. It does not seem likely that the presence of carbohydrate would prevent a mere flushing out of nitrogen. When the dogs were exercised (6), the results in respect to extra nitrogen excretion are similar to these obtained with insulin. The experiments in which the dogs were anesthetized (Table III) demonstrate that the increase in nitrogen cannot be ascribed to the muscular movements or hypertonicity preceding convulsions. Hence it seems probable that these reactions can be attributed to actual metabolic changes.

It has not previously been reported that a condition may be produced in which the injection of insulin causes no change in nitrogen excretion. This result was obtained only if the animals were in a lean condition from a prolonged fast or if they were exercised on the treadmill during the fasting period. This is taken to indicate that the source of the extra nitrogen in the urine is depleted as the fast proceeds. When carbohydrate was given at 4 or 5 day intervals to the otherwise fasting animal there was a definite sparing action on the fasting nitrogen level. As the increase from insulin was obtained up to the 11th day there is probably a sparing action also on the source of the extra nitrogen.

Macleod (7) suggests that in the presence of insufficient carbohydrate, insulin causes the formation of sugar from protein. If

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tissue protein entered into such a reaction one would expect the extra nitrogen output to become increasingly greater as the fast with its accompanying depletion of carbohydrate stores proceeds. Our data show the contrary to occur and indicate that we are dealing here with a store of reserve nitrogenous material or perhaps deposit protein. This substance is spared by carbohydrate, is used probably to form sugar when the demand is sufficient as during insulin hypoglycemia or muscular work, and is exhausted in longer fasting periods. It seems possible therefore that insulin through its hypoglycemic reaction may stimulate a catabolism of nitrogenous material.

SUMMARY.

The urinary nitrogen has been determined for three or more preliminary control hours in 84 experiments. The average deviation from the mean is only ± 2.9 mg. of nitrogen per hour. The hourly nitrogen values range from 28 mg., as the lowest, to 224 mg., as the highest, with 109 mg. as the average.

After short fasting periods of 2 to 4 days the injection of insulin produces a rise in the output of nitrogen in the urine.

Following longer fasting periods no appreciable change in nitrogen excretion is caused by the hormone.

Carbohydrate ingested at intervals during an otherwise fasting period exerts a nitrogen-sparing action. Under these conditions the period of the nitrogen fasting, during which insulin will cause an increased output of nitrogen, is prolonged.

A discussion is given of the interpretation that there is a nitrogen store which is utilized during heavy demands for carbohydrate and which becomes exhausted after periods of prolonged fasting.

BIBLIOGRAPHY.

1. Chambers, W. H., Deuel, H. J., Jr., and Milhorat, A. T., *J. Biol. Chem.*, 1927, lxxv, 423.
2. Nash, T. P., Jr., *J. Biol. Chem.*, 1923–24, lviii, 453.
3. Sokhey, S. S., and Allan, F. N., *Biochem. J.*, 1924, xviii, 1170.
4. Labbé, H., and Théodoresco, B., *Comp. rend. Acad.*, 1925, clxxx, 1438.
5. Hawley, E. E., and Murlin, J. R., *Am. J. Physiol.*, 1925, lxxv, 107.
6. Chambers, W. H., and Milhorat, A. T., *Am. J. Physiol.*, 1927, lxxxi, 469.
7. Macleod, J. J R., Carbohydrate metabolism and insulin, London and New York, 1926.

MUSCULAR EXERCISE AND NITROGEN METABOLISM OF DOGS.*

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Extensive experimental studies concerning the influence of muscle activity on protein metabolism have produced many controversial conclusions since the first paper in 1855. It seems probable that in the large mass of conflicting data on this subject[1] the importance of certain factors has been overlooked. Some of the earlier work was done on fasting animals, in which case the urinary nitrogen is all derived from protein previously accumulated in the body and thus it might vary according to the diet prior to the experiment. Many investigators have maintained individuals on definite diets and compared the 24 hour nitrogen excretion of working and resting days. In such experiments the total output of nitrogen may represent catabolism of some previously stored protein as well as of that ingested. It is an undisputed fact that protein may be utilized for energy (the "dynamic quota" of Rubner). Unless provision is made for the excess caloric requirement on the work days it seems doubtful if experiments in which food is ingested throw light on the question of whether an increase in protein metabolism comes from a "wear and tear" destruction of the muscle due to contraction or represents merely fuel for energy.

* Preliminary reports appeared in *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 170; *Am. J. Physiol.*, 1927, lxxxi, 469.

† Fellow in Medicine, National Research Council, 1924–26, during the first part of the experimental work.

[1] A complete bibliography of this literature prior to 1925 has been carefully reviewed by Cathcart, E. P., *Physiol. Rev.*, 1925, v, 225. The more recent references are given by Cathcart and Burnett (1) and by Mitchell and Kruger (2).

It was shown by Anderson and Lusk (3) that on the 13th day of fasting their dog has a non-protein respiratory quotient of about 0.71 during a 9 mile run, indicating that the work was done entirely at the expense of fat. Unfortunately, the urinary nitrogen could not be accurately followed. In some recent experiments on insulin by the authors (4) it was found that early in fasting an increased metabolism of nitrogenous material was stimulated by the injection of the hormone but after longer fasting periods it had no effect on

PROTOCOL 1.

Dog 124; Experiment 58; weight 12.6 kilos; 3rd day of fast.

End of period.	H_2O ingested.	Exercise.		Urine.	
		Time.	Amount.	Volume per period.	N per hr.
a.m.	*cc.*	*min.*	*kg. m.*	*cc.*	*mg.*
9.50					
10.50	0				123
11.50	0			1	114
p.m.					
12.50	0			1	116
1.42	0			2	115
2.22		30	23,410		
2.33	0			2	153
3.16		30	22,990		
3.28	60			2	156
4.05		30	22,770		
4.15	0			1	179
5.05	40			2	181
5.50	40			1	184

the nitrogen output. In view of the above results it was thought that the state of nutrition of the animal might be an important factor in influencing protein metabolism during exercise. On this basis the experiments here reported were planned.

Methods.

A moderate, steady exercise was obtained by running the animals on a horizontal treadmill driven by a motor at a constant rate of 4 miles per hour. Mongrel female dogs, of a quiet disposition, weighing from 10 to 20 kilos were selected for the experiments

and were readily trained to run within a few days. The urine was collected by catheter for each experimental period as described previously (4). The details of a typical experiment are shown in Protocol 1. After three or four control periods of rest in the metabolism cage, the dog was exercised for three 30 minute runs with 15 minute intervals between the work bouts to allow for catheterization and rest. Postwork observations were also made for about 2 hours. The amount of ingested water was limited to prevent a diuresis and the urine volume was noted. Protocol 1 does not apply to some of the later experiments. When it was found that the nitrogen during exercise and also the resting level changed gradually and regularly from day to day, it was considered unnecessary to divide the control and working periods into three sections each. Thus, without changing the total length of the periods, the catheterizations were reduced to three, one following a 3 hour preliminary rest, one after the three exercise bouts, and one for the postwork excretion about 2 hours thereafter. Total nitrogen in the urine was determined by the Kjeldahl method and all the nitrogen figures are expressed in terms of mg. per hour. In the charts the dotted line represents the nitrogen level of the night urine, and for each day the first horizontal line shows the average of the preliminary control hours, the second horizontal level gives the height of the average for the three work periods, and the third horizontal line is the postwork nitrogen level.

Work is reported in terms of kg. meters, calculated as follows: Work in kg. meters = body weight $\times$ distance run (meters) $\times$ 0.58 kg. meters.

Anderson and Lusk (3) found that an average of 0.58 kg. meters of energy was required per kilo of body weight to move the dog 1 meter horizontally. Inasmuch as our earlier experiments were performed on their treadmill, the factor is applicable.

EXPERIMENTAL.

The main experimental data were obtained from six dogs during eleven different fasting periods which varied from 7 to 37 days. As it was noted in the insulin experiments (4) the basis for calculating changes in nitrogen excretion depends upon the accuracy with which a preliminary control level can be established.

In the thirty-five control periods obtained from the aforemen-

tioned work, the deviation from the mean of three or four determinations averaged ± 2.8 mg. of nitrogen per hour. Confirming these results there are forty-nine control experiments from the data on exercise in this paper. They are divided into two groups, the fasting experiments and those in which sugar was ingested within 24 hours previously. In the former group of twenty-four cases the average deviation was ± 3.8 mg. of nitrogen whereas in the latter group the average was ± 2.2 mg. Thus, carbohydrate exhibits a stabilizing effect on the hour to hour nitrogen excretion as well as a nitrogen-sparing action. Many of the individual figures from which these averages are derived are given in the tables.

TABLE I.

Effect of Exercise on Nitrogen Excretion during Fasting.

Dog 124, Experiment 58.

Day of fast.	Body weight.	Total N per hr. in urine.													Work.	
		Night period.	Prework hrs.					Work hrs.				Postwork hrs.				
			1	2	3	4	Mean and deviation.	1	2	3	Mean.	1	2	Mean.	Time.	Amount.
	kg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*min.*	*kg. m.*
2	13.1			124	108	107	**113 ± 4.3**	142	128	125	**132**	124	114	**120**	90	74,490
3	12.6	102	123	114	116	115	**118 ± 1.8**	153	156	179	**162**	181	184	**183**	90	69,170
4	12.5	135	139	132	127	116	**128 ± 3.5**	154	185	195	**180**	171	192	**180**	90	68,500
5	12.4	125	116	116	144	135	**130 ± 5.9**	148	163	143	**146**	161	172	**166**	90	68,000
6	12.15	109	114	121	117	126	**120 ± 2.0**	131	140	132	**134**	135	140	**136**	90	65,630
7	12.1	96	92	107	101	92	**98 ± 3.0**	122	120	124	**122**	132		**132**	90	67,320
8	11.95	95	106	115	91	97	**102 ± 4.1**	109	122	79	**101**	100	101	**101**	90	68,100
9	11.8	85		111	114	127	**117 ± 3.7**	122	110	100	**110**	110	115	**112**	90	64,430
10	11.7	84	94	113	99	92	**97 ± 3.3**	63	89	97	**84**	97	96	**97**	90	64,000
11*		79														
12	11.5		35	35	43	49	**40 ± 2.8**	41	43	45	**43**	52	48	**50**	90	61,480

* 480 gm. sucrose *per os*.

Experiments on Fasting Dogs.

In the first experiments the dogs were exercised every day during the fasting period beginning on the 2nd day after the last

food ingestion. The results of a typical experiment on Dog 124 are shown in Table I. On the 2nd day of fasting the averages for the preliminary, exercise, and postwork periods show a small increase in protein metabolism only during the work, but on the succeeding days the increased excretion of nitrogen during exercise is unmistakable and is carried on into the postwork period. The height of the extra nitrogen output is reached on the 3rd and 4th days of fasting and then gradually declines until on the 8th, 9th,

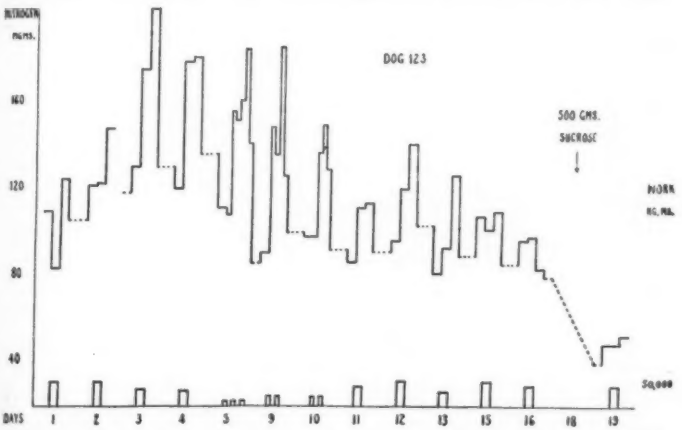

CHART 1. Effect of exercise and fasting on the total nitrogen per hour excreted in the urine. In the charts the dotted line represents the nitrogen level of the night urine, and for each day the first horizontal line shows the average of the preliminary control hours, the second horizontal level gives the height of the average for the three work periods, and the third horizontal line is the postwork nitrogen level.

and 10th days the averages for the work period are the same or less than for the control hours.

Similar results on another dog (No. 123) are illustrated by Chart 1. In this case an hour of rest separated the individual exercise periods on the 5th, 9th, and 10th days of fasting. Contrary to Dog 124 (Table I), during the first bout of work the increase was rarely present but appeared in the rest period following or during the second run on the treadmill. It was not until the 15th and 16th days of fasting that this dog accomplished the exercise without extra nitrogen excretion.

The point at which the extra nitrogen metabolism was exhausted shows a wide variation. For example, in one experiment on Dog 124, running 6 miles produced no increase in nitrogen output on the 6th day, whereas in the observations on Dog 3, shown in Chart 2, this condition was not reached by the 28th day of fasting. On the days noted on the chart the usual amount of work was done, 90 minutes of running on the treadmill. The actual work in kg. meters diminished slightly each day with the decrease in body weight of the animal from 85,600 kg. meters on the 2nd day

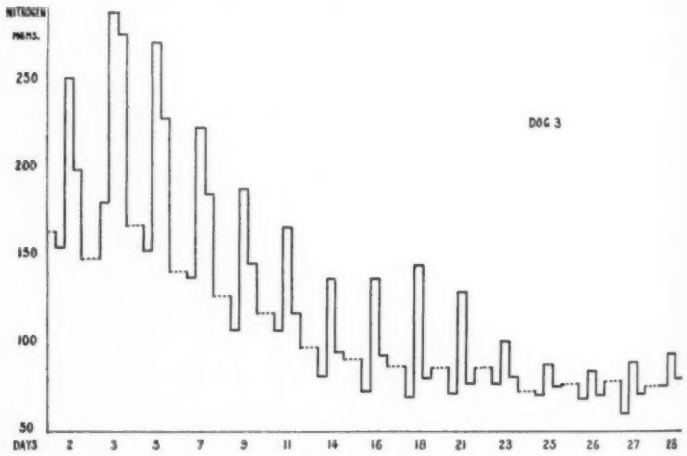

CHART 2. Effect of exercise and fasting on the total nitrogen per hour excreted in the urine.

of fasting to 65,000 on the 28th day. The increase in nitrogen was most marked on the 3rd day, rising from a control level of 181 mg. per hour to 290 during the work and 277 for 3 hours after the exercise. The extra nitrogen gradually fell until the 26th day at which time the control, work, and postwork figures were 69, 84, and 71 mg. respectively. The significance of the slight increase on the last 2 days is discussed later in connection with the other experiments on this dog.

A different type of reaction is exhibited by Dog 2 which exercised

on the treadmill every 2nd or 3rd day, as indicated in Table II, and ran a total of 102 miles during an uninterrupted fast of 37 days. As is noted in Table II, on the 7th day there was fresh blood in the postwork urine which most likely came from an injury of the bladder sphincter. As the blood appeared again at the

TABLE II.

Effect of Exercise on Nitrogen Excretion during Fasting.

Dog 2, Experiment 5.

Day of fast.	Body weight.	Total N per hr. in urine.			
		Night period.	Prework.	Work.	Postwork.
	kg.	*mg.*	*mg.*	*mg.*	*mg.*
2	15.40		206	157	162
3	14.95	128	134	135	187
5	14.55	123	124	138	140
6	14.25	104			
7	14.25	97	104	103	129*
9	13.90		91	118*	168*
12	13.55	80	92	85	86
13	13.35	70			
14	13.30	68	96	89	92
15	13.12	62			
16	13.07	61	90	80	56
18	12.85	62			
19	12.75	70	91	78	82
21	12.47	67	82	66	76
23	12.35	58	89	66	114
26	12.03	57	84	75	89
28	11.85	55	81	60	89
31	11.48	54	81	65	82
34	11.25	54	81	†	68
35	11.22	52	56	45	66
37	11.05	46	84	62	79

* Blood in urine from bladder injury.

† Urinated while running.

second and third catheterizations on the 9th day, the dog was allowed to rest undisturbed until the 12th day. This eliminated the difficulty. Probably after the 7th day, certainly by the 12th day, the exercise was accomplished without extra nitrogen excretion. In fact from the 12th to the 37th day there was a constant although small decrease during the work period.

Experiments on Effect of Carbohydrate.

With the exception that carbohydrate in the form of sucrose was administered at various times to the otherwise fasting animals these experiments are essentially identical with the foregoing ones. In four tests on three different dogs the sugar was given during the early days of the fast. Under these conditions the typical protein-sparing action is evident in both the resting and exercising metabolism. This is illustrated in Table III in the results from Dog 127 when sucrose was ingested daily for 7 days and the exercise followed for 3 days longer. The effect on the resting metabolism is seen in the urines of the prework and of the night periods which cover the time from the last postwork sample of 1 day until the 1st control hour of the following morning. The night and prework nitrogen level during the first 8 days (Table III) was definitely lower than on the 9th and 10th days. The extra nitrogen excretion during exercise also was spared by the carbohydrate. In Table III a slight increase in nitrogen is found in the postwork urines on the 3rd, 5th, and 6th days. This is attributed to an exhaustion of the ingested carbohydrate as the amount given in this experiment was insufficient to maintain the body weight. Other tests show no significant increase in the postwork nitrogen when larger amounts of sucrose were taken.

If carbohydrate is ingested at the end of a fast and work experiment, the nitrogen-sparing activity is evident at this time also. On the 11th day, after the exercise on the 8th, 9th, and 10th days had produced no extra nitrogen (Table I), Dog 124 ingested a total of 480 gm. of sucrose between 11.00 a.m. and 5.15 p.m. The resting nitrogen excretion on the following morning was reduced to one-half of that on the 10th day. The averages for prework, work, and postwork urines are practically the same (Table I). In a similar experiment on Dog 123 the results are in accord with this. On the 18th day of fasting (Chart 1) 500 gm. of sucrose were retained. The figures for the individual hours on the 19th day are exceptionally constant, being 49, 47, and 50 mg. per hour for the preliminary period, 48, 50, and 48 mg. for the 3 work hours, and 53 mg. after the exercise.

Several interesting points are found in the experiment on Dog 3 given in Chart 3. The nitrogen-sparing reaction during the

TABLE III.

Nitrogen-Sparing Action of Sucrose during Exercise.

Dog 127, Experiment 61.

Day of experiment.	Body weight.	Total N per hr. in urine.												Food.
		Night period.	Prework hrs.				Mean and deviation.	Work hrs.				Postwork hrs.			
			1	2	3	4		1	2	3	Mean.	1	2	Mean.	
	kg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
1															100 gm. sucrose.*
2		62		48	62	59	**58 ± 3.0**	62	62	62	**62**	55	79	**65**	170 " "
3	6.8	49		24	25	30	**28 ± 1.8**	38	39	38	**38**	46	44	**45**	170 " "
4		44													200 " "
5	6.6	42	52	52	57	63	**56 ± 2.0**	50	72	82	**61**	79		**79**	100 " "
6	6.5	45	64	62	62	69	**64 ± 1.1**		64	69	**67**	90	90	**90**	100 " "
7	6.3	52	73	64	67	70	**69 ± 1.5**	75		63	**69**	74	78	**76**	100 " "
8	6.2	66	77	66	64	73	**70 ± 2.5**	75	68	69	**74**	71		**71**	None.
9	6.15	80		75	77	70	**74 ± 1.6**	78	71	67	**75**	76	85	**80**	"
10		83		105	91	82	**92 ± 4.7**	83	81	74	**80**	81	86	**84**	"

* The sucrose was given each day after the last postwork urine was collected.

first 4 days is more convincingly demonstrated because on the subsequent fasting days exercise did produce a marked increase. The condition in which work causes no extra nitrogen metabolism was not attained, in this respect agreeing with the previous experiment on the same dog (Chart 1), for after the 11th day each succeeding run on the treadmill *increased* the extra nitrogen in the work and postwork urines. There was a loss in body weight from 14.80 to 11.45 kilos during the 18 days of fasting. The dog was then fed for 30 days on an artificial diet of definite nitrogen content

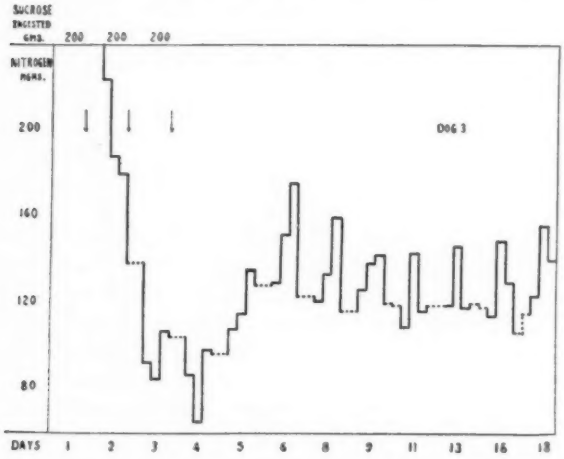

CHART 3. Action of carbohydrate on the total urinary nitrogen per hour during exercise.

made from meat residue, sucrose, lard, butter, bone ash, and salts according to the proportions of Diet IV of Cowgill, Deuel, and Smith (5). Vitamin B was supplied in tomato juice and Vitavose. The total nitrogen intake for 30 days was 339 gm., the output in urine and feces was 292 gm., leaving a positive nitrogen balance of 47 gm. During this time the body weight increased from 11.45 to 13.40 kilos. In the following 2 days of fasting the total nitrogen elimination was 22 gm., leaving a positive balance of 25 gm. at the time the exercise experiment given in Table IV was started. From the table it is seen that the exercise produced a large increase

in nitrogen metabolism which did not diminish during the succeeding days but remained nearly constant for the first 4 days. On the 5th day 200 gm. of sucrose were given at 5.00 p.m. and 100 gm. at 10.30 p.m. The nitrogen-sparing action was only partial, for a comparison of the 6th day with the 4th shows that the prework levels are the same although there was decidedly less increase in the exercise and postwork periods. However the carbohydrate did not entirely prevent the increase of the work level over the control as was the case at the beginning of the experiments on this dog (Chart 3, 2nd, 3rd, and 4th days). To insure a supply of available

TABLE IV.

Effect of Sucrose on Nitrogen Excretion.

Dog 3, Experiment 2.

Days.	Body weight.	Total N per hr. in urine.				Amount of work.	Food.
		Night period.	Prework.	Work.	Postwork.		
	kg.	*mg.*	*mg.*	*mg.*	*mg.*	*kg. m.*	
1	12.80		130	172	171	69,700	0
2	12.55	109	97	167	120	67,500	0
3	12.40	94	73	164	153	67,000	0
4	12.25	81	77	173	163	67,900	0
5	12.10	89					300 gm. sucrose.
6	12.20	105	77	94	96	69,000	0
7	11.85	85					100 gm. sucrose at 10 p.m.
8	11.55	83	75	108	91	64,100	50 " " " 10.30 a.m.

carbohydrate at the time of exercise 100 gm. of sucrose were ingested at 10.00 p.m. on the 7th day and 50 gm. at 10.30 the next morning. Immediately after the sugar was taken the nitrogen excretion for the three rest periods was 77, 72, and 75 mg. per hour and then for the three exercise periods was 107, 108, and 111 mg. per hour. The averages are 75 and 108 respectively, making an increase of 44 per cent. It seems evident therefore that under these conditions carbohydrate was unable to spare completely the protein catabolism during work.

DISCUSSION.

Our data indicate that the nitrogen excretion during exercise can be correlated with the state of nutrition of the animal. In this respect two conditions are outstanding. First, work caused an increased nitrogen output in every case in the early part of the fasting experiments. Second, the same amount of work could be accomplished without any increase in urinary nitrogen after a longer period of fasting and exercising, or after the ingestion of sufficient carbohydrate.

Several of the experiments are suggestive of the significance of this extra nitrogen excretion from muscular exercise. A large increase such as was found in Dog 3, Chart 2, and Dog 123, Chart 1, is not unprecedented in fasting dogs. Frentzel (6) in 1897 published data from a dog which ran on a treadmill on the 3rd, 4th, and 5th days of fasting. His results are shown in the following tabular matter.

Day of fast.	Nitrogen per hour.	
	Work. *gm.*	Rest. *gm.*
1		0.154
2		0.166
3	0.368	0.183
4	0.275	0.196
5	0.240	0.233
6		0.211

He made no note of the fact that the exercise nitrogen per hour diminished each day, probably because this was overshadowed by the increase in the postwork periods. His dog performed about 4 times the amount of work each day that ours did.

In our experiments no nitrogenous food was given throughout the time that the exercise was being observed, therefore the complicating factor of exogenous origin is eliminated. The data seem to favor the interpretation that the extra nitrogen is a true product of metabolism and not merely the result of a flushing out process of accumulated inert nitrogenous material on account of the increased blood flow accompanying the exercise. In so far as could be observed, the physical differences between rest and work were present to the same degree on the 10th as on the 4th day of exercise (Table I); nevertheless, the extra nitrogen excretion

amounted to 0 and 41 per cent respectively. The fact that the extra nitrogen can be replaced by carbohydrate (Chart 3) is additional evidence for its metabolic origin. In this respect also the results from injecting insulin should be cited (4). It was found that insulin caused an increase in nitrogen excretion early in the fasting period but did not do so after a prolonged fast or if sugar was given with the insulin. Thus a similar reaction of extra nitrogen is produced by two separate sources, muscular work and the injection of insulin.

All the experiments with the exception of two, which were on the same dog, show that the extra nitrogen is exhausted after a time, therefore indicating that it may come from the catabolism of a nitrogen store or deposit protein and not from the muscle tissue itself. The recent investigations of Cathcart and Burnett (1) show that the extra nitrogen which they obtained was mainly urea nitrogen as no significant changes in creatinine or uric acid nitrogen were found. The sulfur:nitrogen ratio of the *extra* nitrogen and sulfur was about 1:27 for three of the diets and was similar to that for muscle tissue, *i.e.* 1:14, only when meat was taken. Thus their findings suggest that the extra nitrogen may come from a source other than the muscle tissue. Mitchell and Kruger (2) also report no increase in creatinine excretion in exercising rats which are fed on an adequate diet.

Attention should be directed in this connection to the data in Chart 3 and Table IV from the dog that showed toward the end of the experiments an increase instead of a decrease in extra nitrogen. Carbohydrate could completely replace the extra nitrogen early in the fasting period but at the end of the experiment was unable to do so, or to cause the usual decrease in the resting nitrogen level. The lean condition of the dog may help to explain this exceptional reaction. Prior to the experiment an attempt was made to keep the dog on a synthetic food but as it ate only 470 gm. in 7 days the diet was changed. After 4 days of an abundant ration containing a surplus of meat, the experiment shown in Chart 3 was conducted. It is thought that the increase in extra nitrogen after the 11th day may represent a demand upon tissue protein due to the depletion of body fat as the dog was extremely emaciated. During the succeeding period of feeding, the 25 gm. of nitrogen retained are about equal to the total excretion between the 10th

and 18th days. Over the same periods of time the loss and subsequent gain in body weight are equal, thus it seems doubtful whether the dog was able to store much fat during the feeding period. Hence the high and undiminishing extra nitrogen excretion for the first 4 days shown in Table IV probably represents some tissue protein catabolism also. The nitrogen-sparing action of sugar was not complete at this time (Table IV) as it was at the beginning of the experiment (Chart 3), suggesting a difference in the source of the extra nitrogen in these two instances. In another experiment on this dog (Chart 2) the initial body weight was higher, 15.0 kilos. In this case the extra protein supply was much larger, lasting for 26 days, during which time the body weight fell to 11.67 kilos, then a slight rise in exercising nitrogen was encountered similar to that in Chart 3.

The extra nitrogen from work appears to be spared by body fat if we compare the results from Dog 3, Chart 2, with those from Dog 2, Table II, although not to the same extent as by carbohydrate. The former, a lean dog, produced extra nitrogen for 28 days, whereas the latter, a very fat one, exercised from the 12th to the 37th day without extra protein catabolism. This is in agreement with the early work of von Voit (7) on the difference in results from young, lean dogs, and old, fat ones.

In 1908 Lusk (8) exercised a phlorhizinized dog on the 3rd day of diabetes and the 6th day of fasting. On the preceding day the dog ran for 900 meters and then shivered for 3 hours at 10°. The exercise results follow:

2 Hour Urine Samples.

Period.	Dextrose. gm.	Nitrogen. gm.	D:N
Rest	4.20	1.19	3.53
1500 m. run	5.32	1.36	3.90
Rest	4.57	1.26	3.63
1500 m. run	4.62	1.26	3.67

If the figures for the first exercise period are compared with the following rest hours, it is seen that the work produced an extra excretion of 0.75 gm. of dextrose and 0.10 gm. of nitrogen. The ratio of this extra D and N is 7.50 or twice the ratio of tissue protein or of ingested meat. The second bout of work had no significant effect on either the nitrogen or sugar excreted.

From the above considerations it is thought that the extra nitrogen eliminated on exercise comes from the catabolism of a nitrogen store available for energy. This nitrogen store can be fully replaced by carbohydrate, be called forth when the demand for carbohydrate in the body is increased as it is in exercise or insulin hypoglycemia, and be exhausted by prolonged fasting and exercise.

The second condition noted above, namely that muscular exercise can be accomplished without an increase in urinary nitrogen, is probably the more important one. Much emphasis was placed in the early discussions on the possibility of the increase appearing on the following day. There is no evidence in our data that such occurs. On the 13th and 15th days in Table II the night urine containing the complete 24 hour sample following an exercise day is lower in nitrogen instead of higher than the work urine. In the fasting experiments, Dog 2 (Table II) exercised on 11 different days between the 12th and 37th days of fasting without any increase in urinary nitrogen. The same applies to Dog 124 (Table I) on the 8th, 9th, and 10th days and to Dog 123 (Chart 1) on the 15th and 16th days. In addition to this we have observed eleven similar results on four other dogs. When sufficient sugar was given, the work was accomplished without extra nitrogen metabolism. Eighteen trials on five different dogs substantiate this conclusion. Disregarding interorganic changes in nitrogenous substances within the body, concerning which urinary determinations will show nothing, these data are in favor of the conclusion that moderate muscular work can be performed without a destruction of tissue protein.

SUMMARY.

The effect of muscular work on the total urinary nitrogen excretion has been studied in dogs that were exercised moderately on a horizontal treadmill.

In the prework control periods the average deviation from the mean of three or four determinations is ± 2.9 mg. of nitrogen per hour on the basis of 84 experiments. The range of the means is from 28 ± 1.8 to 224 ± 6.3 mg. per hour, hence the average of 3 or 4 resting hours establishes a nitrogen level with an average accuracy of about 3 per cent.

An increase in nitrogen metabolism of as much as 50 to 100 per cent is produced by work on the 3rd or 4th day of fasting. This is prolonged into the postwork period.

The extra nitrogen from work can be completely spared by carbohydrate. It gradually disappears as the fast is continued.

These data and similar results following the injection of insulin suggest that the extra nitrogen may come from an exhaustible reserve rather than from tissue protein.

Muscular work in the dog can be accomplished without any increase in protein metabolism in so far as it is possible to determine from the excretion of urinary nitrogen.

BIBLIOGRAPHY.

1. Cathcart, E. P., and Burnett, W. A., *Proc. Roy. Soc. London, Series B*, 1925–26, xcix, 405.
2. Mitchell, H. H., and Kruger, J. H., *J. Biol. Chem.*, 1928, lxxvi, 55.
3. Anderson, R. J., and Lusk, G., *J. Biol. Chem.*, 1917, xxxii, 421.
4. Milhorat, A. T., and Chambers, W. H., *J. Biol. Chem.*, 1928, lxxvii, 595.
5. Cowgill, G. R., Deuel, H. J., Jr., and Smith, A. H., *Am. J. Physiol.*, 1925, lxxiii, 106.
6. Frentzel, J., *Arch. ges. Physiol.*, 1897, lxviii, 212.
7. von Voit, C., in Hermann, L., Handbuch der Physiologie, Leipsic, 1881, vi, 189.
8. Lusk, G., *Am. J. Physiol.*, 1908, xxii, 163.

STUDIES ON LIPOCHROMES.

III. THE QUANTITATIVE ESTIMATION OF CAROTIN IN BLOOD AND TISSUES.

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It has now been well established that carotin, one of the lipochrome series of pigments, is almost constantly present in the blood. Van den Bergh and Snapper (1), in 1913, distinguished the lipochrome of cattle serum from bilirubin, and showed that the pigment in the skin in the "xanthosis diabetica" of Von Noorden (2) was associated with a similar pigment in the blood plasma. Palmer (3) described the lipochrome of horse serum, and identified it as the carotin ingested with the food. Palmer and Eckles (4) also showed carotin to be the lipochrome of cow fat, milk, and blood, and found this same substance to be the natural yellow pigment of human milk. Another lipochrome, xanthophyll, was found by Palmer and Kempster (5) to be the principal pigment of chicken skin, blood, and egg yolk, and Palmer (6) has found both carotin and xanthophyll in human blood. Willstätter and Escher (7) identified the pigment of the corpus luteum of cattle as carotin, and Van den Bergh, Muller, and Broekmeyer (8) have extracted these pigments from human blood and tissues, and from the organs of various animals.

Carotinemia has been known since Van den Bergh and Snapper's paper, but Hess and Myers (9), in 1919, first applied that name to the condition in which an excess of lipochrome appeared in the blood as a direct result of a high vegetable diet. Umber (10) noted the same condition in 1916, and Bürger and Reinhart (11), and Salomon (12) showed the pigment to be of exogenous origin. A large number of cases were reported in Germany, in 1919, occurring in diabetics, in children under asylum conditions, or in adults upon the semistarvation diet during the war (13). Head and Johnson (14) have reported a single case of carotinemia in a diabetic, and Stoner (15), recently, another. Hashimoto (16) reported thirty-five cases in Japanese upon a heavy squash diet, and quoted Baelz as recording similar cases in 1896 under the term "aurantiasis cutis."

Palmer and Kempster showed that xanthophyll disappeared from the blood, skin, and egg yolk of chickens when fed a xanthophyll-free diet, and in the recorded cases of carotinemia the condition has disappeared upon change of diet to one containing less green and yellow vegetables. Dolly and Guthrie (17) found that certain nerve cells of chickens did not contain

lipochrome when none was present in the diet, and that, on the contrary, the pigment did appear in nerve cells when a lipochrome-rich diet was fed. It seems to be well established, therefore, also, that the lipochrome present in the animal body has been derived from the vegetable content of the food. It is noteworthy, however, that all cases recorded in which there has been an excess of pigment in the skin and blood have been among diabetics, or children or adults upon an otherwise deficient diet. It is also noteworthy that many diabetics have a yellowish coloration of the skin even when, by the use of insulin, they are not upon an excessively high vegetable diet (18).

Van den Bergh found that the carotin in the blood could not be directly extracted with petroleum ether, though the pigment is readily soluble in this substance. Palmer showed that the pigment is closely bound to the protein of the blood, and upon fractional precipitation it was found to come down with the euglobulin. It could be released from this union by the addition of 95 per cent alcohol to the plasma, after which it was easily extracted by lipoid solvents. But in a few cases of diabetes Palmer found that such a union did not exist; the carotin could be extracted directly with petroleum ether.

Willstätter and Stoll (19) proposed a test for carotin using 0.2 per cent potassium dichromate as a standard against carotin dissolved in petroleum ether. This standard is equal to a 5×10^{-5} molar solution of carotin equivalent to a 0.00268 per cent solution. This is not strictly quantitative, however, as they found 101 mm. of potassium dichromate solution in the colorimeter to equal 100 mm. of carotin solution, 41 mm. to equal 50 mm. of carotin, and 19 mm. to equal 25 mm. of carotin. This standard was used for the determination of the carotin content of leaves in which the pigment is relatively abundant. Palmer adapted this to the estimation of carotin in blood by treating the serum with an equal amount of 95 per cent alcohol, mixing with plaster of Paris, then adding petroleum ether. The method is useful for large amounts of blood, but cannot be used very well with the small amounts available in the clinical laboratory. Van den Bergh, Muller, and Broekmeyer used 1 to 2 cc. of serum, added an equal amount of alcohol, then extracted with petroleum ether. They used $\frac{1}{2}\frac{1}{4}$ per cent solution of potassium dichromate as a standard. They also estimated the amount of carotin and xanthophyll in the organs of various animals, but they do not record their method for the extraction of the latter.

While studying the reaction of animals when injected or fed upon carotin it became necessary to determine the carotin content of the blood and organs of these animals (20). I was, at the same time, attempting to distinguish the various pigments present in human organs (heart muscle, seminal vesicles, *etc.*) by histological methods, and as a check, a chemical examination of these organs was desirable. The carotin content of such tissues promised to

be exceedingly minute, and, as a consequence, an investigation of the standard used by Willstätter and Stoll seemed necessary.

It was first found that progressive dilution of the standard potassium dichromate solution did not equal an equivalent dilution of carotin. When the concentration was very low, a 0.2 per cent solution would no longer be accurate. So a series of tests was made with carotin freshly prepared according to a method previously described. The resultant carotin in petroleum ether showed typical absorption bands in the spectroscope. A solution containing 2.68 mg. per cent was made and compared with 0.2 per cent potassium dichromate solution. This was found to equal 103 per cent of the calculated amount from Willstätter and Stoll's figures. From this solution the range of colors with carotin in

TABLE I.

Relation of Carotin to Different Concentrations of Potassium Dichromate Solution, at 30 Mm. Depth, in the Colorimeter.

Potassium dichromate solution.	Carotin solution.
per cent	*per cent*
0.4	0.00462
0.2	0.00268
0.1	0.00119
0.05	0.00052
0.04	0.00033
0.025	0.00016
0.02	0.00010

various concentrations was determined as shown in Table I. It was found that the 0.02 per cent solution was the most generally useful.

Methods of Procedure and Results.

It was found (in about 60 per cent of the cases) that 2 cc. of blood serum would yield a visible pigment when extracted with petroleum ether after treatment with alcohol. In the other 40 per cent the pigment was either absent or present in an amount insufficient to measure. The use of whole blood reduced the proportion about half, and reduced also the yield per 100 cc., when present at all in measurable amount. It was found that 3 cc. of

plasma were the least amount which could be depended upon to yield a measurable amount of pigment. The test is simple: 3 cc. of 95 per cent alcohol are added to 3 cc. of plasma in a test-tube, the mixture shaken, and allowed to settle until solid. If excess alcohol rises to the top it may be poured off. 4 cc. of petroleum ether are added, the tube corked immediately, and shaken vigorously for a minute. It is allowed to settle until the petroleum ether rises to the top. This is poured into the colorimeter cup, calculated as 4 cc., and compared with 0.04 per cent or 0.02 per cent potassium dichromate solution equalling, respectively, 0.0003 per cent and 0.0001 per cent of carotin.

For the estimation of the pigment content of organs a much different procedure is necessary. Macerating the tissue in 95 per cent alcohol then extracting the crushed material, alcohol included, in a continuous Soxhlet extractor is effective but time-consuming. It was found that a quantitative extraction could be obtained by the following method. The weighed organ was placed in an excess of 20 per cent KOH solution in 70 per cent alcohol, and boiled until dissolved. The solution was then mixed with enough $CaSO_4$ to take up the water, and direct extraction with petroleum ether performed. The extract was washed with several portions of 85 per cent alcohol until no more color could be removed. The petroleum ether fraction was considered to be carotin, and the alcohol to contain xanthophyll, if present. The alcohol was mixed with ether (diethyl ether) after the method of Willstätter and Stoll, and the alcohol washed out with distilled water. The xanthophyll was thus transferred to the ether, and the pigment which remained in the alcohol-water fraction, if any, was discarded. The petroleum ether solution was made up to a definite amount and compared in the colorimeter against potassium dichromate solution. The xanthophyll was not determined quantitatively as it was present in extremely small amounts or not at all in all tissues examined except chicken fat. The results of these determinations and of blood under various conditions are as follows:

Blood.—Thirty-six specimens obtained from the Massachusetts State Wassermann laboratory, condition of patients unknown; serum: 0.02 to 0.11 mg. per cent. None present in twelve specimens.

Three specimens from normal adults; plasma: 0.04, 0.07 mg. per cent, and none.

Eighteen specimens from diabetics under the care of Dr. E. P. Joslin;[1] plasma: 0.05 to 0.16 mg. per cent. Three specimens contained too little to measure.

One specimen of normal plasma: 0.05 mg. per cent; plasma 2 hours after ingestion of 50 mg. of carotin in olive oil: 0.08 mg. per cent; 4 hours after ingestion of carotin: 0.04 mg. per cent.

Van den Bergh, Muller, and Broekmeyer found 0.4 to 1.34 mg. per cent in normal blood, and 0.45 to 1.9 mg. per cent in diabetic blood.

Organs.—Heart muscle freed from fat and blood: (1) From a 44 year old woman; very faint trace of carotin; (2) from a 79 year old man; heart weight, 280 gm.; no carotin; (3) from an 81 year old man; heart weight, 340 gm.; no carotin; (4) from a 3 month old infant; heart weight, 21 gm.; no carotin. Adrenals, from eight adults, various ages; carotin 4.25 to 15.6 mg. per cent. Adrenals, from a 3 month old infant; no carotin. Liver from four adults, various ages; carotin 1.0 to 6.0 mg. per cent. Liver from 3 month old infant; no carotin. Spleen from four adults; carotin, a trace to 2.1 mg. per cent. Spleen from infant; no carotin. Fat from an obese elderly woman; carotin 7.5 mg. per cent. Seminal vesicles from three adults, two quite old; no carotin. Testicle from 79 year old man; no carotin. Corpus luteum from a surgical specimen; carotin 4.1 mg. per cent; also contained an alcohol-soluble pigment, possibly xanthophyll.

Guinea Pig Tissues.—Heart, spleen, testes, blood of a normal animal; no carotin. Adrenal from a normal animal; carotin 0.4 mg. per cent. Liver from a normal animal; carotin, a trace. Blood, bile, and urine from an animal 5 hours after feeding carotin in olive oil; no trace of carotin. Blood and urine after the intraperitoneal injection of about 300 mg. of carotin; no trace of pigment. Blood, spleen, kidneys, testes, adrenals of an animal which had been on a carotin-free diet; no carotin. Feces of a guinea pig which had been on a synthetic diet for about 3 years, receiving 5 cc. of orange juice a day; carotin 0.01 mg. per cent. (A sample of orange juice contained 0.04 mg. per cent of petroleum ether-soluble material, calculated as carotin, and 0.14 mg. per cent of alcohol-soluble pigment, calculated as xanthophyll.) Feces of a normal guinea pig on a carrot diet; carotin 7.0 mg. per cent.

Other Tissues.—Normal rabbit: fat, blood, spleen, testes, none; liver, a trace; adrenals, from two animals; 0.2 and 0.3 mg. per cent. Pork fat, mutton fat, beef fat, none. Chicken fat contained considerable yellow pigment which was soluble in 85 per cent alcohol and was therefore considered to be xanthophyll.

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[1] Secured through the courtesy of Miss Hazel M. Hunt, Head of the Clinical Laboratory, Deaconess Hospital, Boston.

DISCUSSION.

It is generally asserted by text-books of pathology (MacCallum, Karsner, Wells) and in the literature on the subject (review by Oberndorfer (21)) that lipochromes are present in the adrenal cortex, corpus luteum, seminal vesicles, testes and epididymi, sympathetic ganglion cells, nerve cells of the central nervous system, liver, heart, muscle of the intestine, pigmented cells of the pineal body, kidney, spleen, fat, skin, sebaceous glands, perineural and perivascular sheaths. Most of the work supporting these reports has been based upon histological methods, and has been dependent upon the assumption that all pigments which are associated with fats, as determined by staining methods, are lipochromes. I have shown previously (22) that most of the methods heretofore used for the differentiation of these pigments cannot be depended upon; that, in fact, lipochrome does not stain with fat stains, and that other crystalline pigments may adsorb some of the fat stains on their surfaces, and so give the appearance of having been stained.

Most of the tissues enumerated above are present in the body in so small amount that they are not amenable to chemical analysis. But liver, heart, spleen, adrenal, fat, corpus luteum, and seminal vesicle tissues were obtained in comparative abundance. Of these only the adrenals, liver, and corpus luteum showed definite measurable amounts of lipochrome. The seminal vesicles, which in all instances contained a generous amount of brownish pigment, contained no carotin or xanthophyll, and likewise two hearts, both fairly typical examples of the condition known as brown atrophy, were negative. The amount of pigment found in the spleen was in no greater amount than could be expected to be present in the contained blood.

The results with normal blood show that this pigment is not present in measurable amount at all times. Here it may be explained that the figures given by Van den Bergh are not at great variance with those recorded above. This writer probably used $\frac{1}{24}$ per cent potassium dichromate solution to equal approximately 0.2 of Willstätter and Stoll's standard 0.00268 per cent carotin solution, or 0.0005 per cent, whereas I have shown that a 0.04 per cent solution of potassium dichromate equals not 0.0005 per

cent but 0.0003 per cent carotin. My figures, therefore, with use of a standard about half as strong as Van den Bergh used equal about half of his calculated results. It is quite possible, however, that more vegetables, and therefore more carotin, are eaten by the average person in Holland than in this country. It is shown that the carotin content of the blood rises slightly after the ingestion of carotin in olive oil.

The diabetic blood shows slightly but consistently higher figures than normal blood. Many of these cases were upon what may be regarded as essentially normal diets. In a few cases there was also a moderate lipemia, and it is possible that these two conditions, lipemia and hypercarotinemia, may be associated. It remains to be determined, however, how constant such an association may be.

SUMMARY AND CONCLUSIONS.

A method, essentially a modification of that used by Van den Bergh, Muller, and Broekmeyer, is proposed for the quantitative estimation of carotin in small amounts of blood. A similar method is devised for the estimation of carotin and xanthophyll in organs and tissues. Various concentrations of potassium dichromate were tested against known amounts of carotin for the purpose of securing accurate standards for comparison when extremely small amounts of pigment were present in blood or tissues. By the application of these methods it was found that carotin is frequently but not constantly present in normal blood; that it rises slightly in amount after the ingestion of carotin in olive oil; and that it is present in slightly greater amounts than usual in diabetic blood, sometimes associated in these cases with a lipemia.

Lipochromes are constantly present in the adrenals of adults, but not present in these or other organs of infants; they are present also in the corpus luteum, liver, and fat, but were not found in recognizable quantities in other organs except the spleen. The amount found in this last organ could be accounted for by that present in the contained blood. The seminal vesicles and heart, in all cases well colored, did not contain pigments demonstrable by the methods used. The adrenal glands of rabbits and guinea pigs contain the greatest amount of lipochrome in these animals; next to these organs the liver contains the most. There is no caro-

tin present in the blood of these animals even after the feeding or injection of comparatively large amounts.

BIBLIOGRAPHY.

1. Van den Bergh, A. H., and Snapper, J., *Deutsch. Arch. klin. Med.*, 1913, cx, 540.
2. Von Noorden, C., Die Zuckerkrankheit, Berlin, 4th edition, 1910.
3. Palmer, L. S., *J. Biol. Chem.*, 1916, xxvii, 27.
4. Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191, 211, 223, 237, 245.
5. Palmer, L. S., and Kempster, H. L., *J. Biol. Chem.*, 1919, xxxix, 299, 313, 331.
6. Palmer, L. S., Carotinoids and related pigments, New York, 1922.
7. Willstätter, R., and Escher, H. H., *Z. physiol. Chem.*, 1912, lxxvi, 214.
8. Van den Bergh, A. H., Muller, P., and Broekmeyer, J., *Biochem. Z.*, 1920, cviii, 279.
9. Hess, A. F., and Myers, V. C., *J. Am. Med. Assn.*, 1919, lxxiii, 1743.
10. Umber, *Berl. klin. Woch.*, 1916, liii, 829.
11. Bürger, M., and Reinhart, A., *Deutsch. med. Woch.*, 1919, xlv, 430.
12. Salomon, H., *Wien. klin. Woch.*, 1919, xxxii, 495.
13. Kaupe, W., *Münch. med. Woch.*, 1919, lxvi, 330. Stoelzer, W., *ibid.*, 1919, lxvi, 419. Klose, E., *ibid.*, 1919, lxvi, 419. Moro, E., *ibid.*, 1919, lxvi, 674. Salomon, H., *ibid.*, 1919, lxvi, 564. Schüssler, *ibid.*, 1919, lxvi, 596.
14. Head, G. D., and Johnson, R. A., *Arch. Int. Med.*, 1921, xxviii, 268.
15. Stoner, W. C., *Am. J. Med. Sc.*, 1928, clxxv, 32.
16. Hashimoto, H., *J. Am. Med. Assn.*, 1922, lxxviii, 1111.
17. Dolly, D. H., and Guthrie, F. V., *J. Med. Research*, 1919, xl, 295.
18. Gordon, A. H., Connor, C. L., and Rabinowitch, I. M., *Am. J. Med. Sc.*, 1928, clxxv, 22.
19. Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll, Berlin, 1913.
20. Connor, C. L., *Am. J. Path.*, 1928, in press.
21. Oberndorfer, S., *Ergebn. allg. u. path. Anat., 2. Abt.*, 1921, xix, 47.
22. Connor, C. L., *Am. J. Path.*, 1928, in press.

SULFUR METABOLISM AND PARTITION OF SULFUR IN THE URINE OF FASTING DOGS.

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On the assumption that all sulfur and nitrogen present in the urine are derived from protein metabolism, it is to be expected that there would be a close parallelism between the excretion of the two, and, furthermore, that the two would be in the same proportion as in the protein undergoing catabolism. In fact, von Wendt (15) expressed the view that no true picture of protein metabolism could be obtained unless the excretion of both nitrogen and sulfur in the urine was followed simultaneously. Together with a number of other investigators, he assumed that the sulfur-containing moiety of the protein molecule is more labile than the sulfure-free fraction, in order to account for the variations in the N:S ratio observed in feeding experiments.

During fasting, when all protein metabolism is endogenous, the N:S ratio, at least theoretically, would be the same as that of the protein catabolized. The ratios actually noted in several careful investigations on fasting humans (1, 3, 9, 13) indicate that during fasts ranging from a few days to 31 days the average N:S quotient varies between 15 and 17. Considering that for muscle tissue, which during inanition is drawn upon extensively, the N:S ratio is 13.3, this consistent discrepancy suggests that the higher urinary ratio may be due to the fact that other tissue proteins with a lower sulfur content are also being metabolized. It seemed desirable to study the changes in the urinary N:S ratio during prolonged fasting with the view in mind of possibly deriving a clue as to the nature of the tissue protein predominantly catabolized at different stages. Furthermore, the urinary sulfur, like urinary

nitrogen, appears in a variety of forms which can be grouped in three fractions, as the salts of sulfuric acid, and as organic combinations of sulfuric acid, in both of which the sulfur is completely oxidized, and finally as neutral or unoxidized sulfur. The latter fraction, of course, is made up of a variety of sulfur-containing substances. With the exception of Cathcart's work on a short fast with a human subject, the partition of sulfur during prolonged fasting has not been undertaken. The behavior of this last fraction, the unoxidized sulfur, during fasting presents further interest in that it may be a measure of the oxidative process during inanition.

EXPERIMENTAL.

Procedure.

The dogs were kept in metabolism cages. The animals were thoroughly washed and cleaned before being put into the cages. The urine was taken daily with a catheter and was rarely voided directly in the cage. At the closing of each period the bladder was irrigated so that every trace of urine belonging to each period was utilized. The dogs drank distilled water.

The nitrogen was determined by the Kjeldahl method. The sulfates were determined by Fiske's benzidine method except in the case of Dog 2, where the determinations were made by Folin's procedure of precipitation with $BaCl_2$. The urines were preserved with a 10 per cent thymol solution in chloroform. This is an excellent preservative for urine and repeated pH determinations have shown that its reaction remained unchanged over a long period of time, so that no bacterial action has taken place.

In the experiments with Dogs 6, 7, 9, 10, and 11, the same food was used during the preliminary and refeeding periods. We employed a modification of the Cowgill diet (4), fresh meat being used as a source of protein. We have not been successful with either casein or meat residue as recommended by Cowgill, the animals refusing to take this food over any length of time. We have used beef heart from which the elastic and fatty tissue had been carefully trimmed away. The meat was ground finely and washed overnight in running water to remove the blood. The washed meat was then squeezed dry in a press. Weighed amounts of the meat packed into solid cakes were wrapped in waxed paper

and stored in a metal can placed in the brine of the University refrigerating plant. The meat was thus frozen solidly and preserved for use in a number of experiments. Samples of the beef heart supply thus prepared were analyzed for moisture, nitrogen, and sulfur, the latter being determined in the dry residue. For the sulfur determination a weighed amount of the dry meat powder was dissolved in nitric acid, evaporated to dryness, and the residue treated with Benedict's sulfur reagent. The sulfur was determined by both the benzidine and the $BaCl_2$ methods. A part of the beef heart supply contained 2.85 per cent N and 0.132 per cent S; in another batch we found 2.86 per cent N and 0.139 per cent S of the fresh material. This gives an N:S ratio in the food of 21.6 and 20.6 respectively. Our results for sulfur are lower than those obtained by Lewis in his analysis of beef heart (0.191 per cent S). In a study of the mineral composition of various organs[1] the total sulfur content of heart is given as 0.154 per cent, with 0.032 per cent in the form of sulfate (SO_4). This would leave 0.143 per cent S probably of protein origin, a value not far removed from the sulfur content of the beef heart supply used in our experiments. Inasmuch as the beef heart was thoroughly washed in running water, it is probable that it had lost practically all of its sulfate, hence, the relatively low sulfur content of the material as compared with that analyzed by Lewis (11).

We have no analysis of the food which was used in experiments with Dogs 2 and 5, and the dietary as far as the protein supply is concerned was not as uniform as in the case of the other dogs. This, however, will be discussed in detail in conjunction with the description of each metabolic experiment.

Results.

Dog 2 (Table I) fasted 36 days and lost 33.6 per cent in weight. During the preliminary period it was fed the regular Cowgill

[1] Unfortunately I am unable to find the exact reference to the paper from which these data were taken. I have copied the table giving the Na, K, Ca, Fe, Mg, Cl, total P, PO_4, total S, and SO_4 of the blood, heart, liver, kidney, spleen, brain, and lungs. The bibliographic card attached to this table became separated and lost, and at the time I discovered this my recollection of the original source became so dim that I was no longer able to return to it in spite of strenuous efforts on my part.

food mixture containing "meat residue." On the basis of analysis of meat residue as reported by Fay and Mendel (7) the dog consumed during the preliminary period 34.9 gm. of nitrogen and 2.21 gm. of sulfur. The urinary excretion for the same period was 28.0 gm. of N and 0.947 gm. of S, giving a N:S ratio of 29.6. Obviously, there had been a retention of sulfur which was responsible for this high N:S ratio. With the progress of the fast the N:S ratio gradually diminishes, dropping to 19.8 in the first fasting period and remaining practically constant (16.6) during the last three or four periods. Assuming that the sulfur retained during the preliminary period was eliminated in the course of the fast, the average urinary N:S ratio for the entire experiment would increase from 17.6 to 25.5, but even the lower value for the N:S ratio indicates that more nitrogen than sulfur is being catabolized during the fast than would be expected if both were derived exclusively from muscle protein.

The changes in sulfur partition are interesting. The inorganic sulfates during the preliminary period constituted practically 78 per cent of the total, but during the fast the ethereal sulfates in this animal attain an unusually high value and this is partly responsible for the diminution of the inorganic fraction which, however, maintains itself fairly constant at about 63 per cent of the total S. The neutral sulfur fraction shows a considerable rise immediately upon the commencement of fasting but diminishes during subsequent fasting periods. It remains then more or less constant throughout the fast.

Dog 5 (Table II) refused to eat the regular Cowgill mixture and it was necessary to supply protein in the form of fresh meat. The food contained 12.08 gm. of N per day but unfortunately no actual analysis was made for sulfur. From available analyses of meat, however, this may be estimated at 0.21 per cent S, giving a N:S ratio in the food of 14.4. During the preliminary period there has been considerable retention of sulfur, quite out of proportion to the possibly small positive N balance in the urine. This manifests itself in the high N:S quotient which is 22.6 for the preliminary period.

The dog fasted 24 days without water, losing weight rather rapidly, so that 34 per cent of the body weight was lost by the end of the experiment. The urinary N:S ratios during the fast are

very high, increasing gradually through the first four periods but diminishing in the remaining two periods. These high ratios indicate that the dog eliminates relatively too much nitrogen which cannot be attributed to a retention of sulfur when one takes into consideration the long period of time over which this experiment extended. The high N:S ratio seems to be a metabolic peculiarity of this dog, becoming even more accentuated during the refeeding periods. We have already mentioned that we could not induce this dog to take Cowgill's synthetic diet and on breaking the fast it was found necessary to feed the mixture made up in

TABLE I.

Dog 2, female collie; 13.05 kilos.

Period.	Duration.	Change in weight.	Total N.	Total S.	N:S ratio.	S.			Per cent of total S.		
						Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.
	days	*per cent*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*			
Preliminary.			28.00	0.947	29.6	737	42	168	77.9	4.4	17.7
Fasting.											
1	4	−6.8	13.52	0.701	19.8	452	63	186	64.5	9.0	26.5
2	4	−11.8	11.11	0.635	17.5	397	102	136	62.6	16.0	21.4
3	4	−15.7	8.47	0.453	18.7	299	61	94	65.9	13.5	20.6
4	4	−19.4	10.08	0.545	18.5	340	83	122	62.4	15.2	22.4
5	4	−22.2	7.79	0.451	17.3	274	72	105	60.8	16.0	23.2
6	4	−25.5	7.50	0.438	17.1	308	33	97	70.1	7.6	22.3
7	4	−28.2	7.23	0.432	16.7	216	93	123	49.9	21.5	28.6
8	4	−30.7	6.60	0.408	16.2	264	57	87	64.6	14.2	21.2
9	4	−33.6	6.50	0.396	16.4	247	70	79	62.4	17.6	20.0

milk. After the first refeeding period, however, the dog ate willingly the straight Cowgill mixture with casein as a source of nitrogen. On the basis of Fay and Mendel's results, we assume a N:S ratio of 16.3 for casein. The nitrogen and sulfur intake during the first refeeding period has been estimated on the basis of this available analysis. From this we find that about 10 gm. of N were retained during that period. The nitrogen intake during succeeding refeeding periods was increased and there was a correspondingly greater N balance at successive periods. But even

XUM

allowing 5 per cent loss of the total nitrogen intake in the feces, the nitrogen retention during 11 days of refeeding is still in the ratio of 6.5:1 to the retained sulfur. In other words, much more sulfur had been retained than would be necessary for the synthesis of new tissue protein. We find likewise that the N:S ratio in the urine during refeeding is unusually high (35.3 to 57.9), thus further corroborating the excessive sulfur retention. The period is too long to justify the assumption that this very high N:S ratio in the urine is due to lag in the sulfur excretion. Taking the average

TABLE II.

Dog 5, female collie; 19.0 kilos.

Period.	Duration.	Change in weight.	Total N.	Total S.	N:S ratio.	S.			Per cent of total S.		
						Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.
	days	*per cent*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*			
Preliminary.	4		43.95	1.940	22.6	1645			84.8		
Fasting.											
1	4	−8.5	18.38	0.704	26.1	605			86.0		
2	4	−14.3	16.15	0.576	28.0	475			82.5		
3	4	−19.1	15.58	0.550	28.3	446			81.1		
4	4	−24.1	16.66	0.514	32.3	392			76.2		
5	4	−28.9	15.06	0.550	27.4	414			75.3		
6	4	−34.0	13.16	0.574	23.0	407			70.9		
Refeeding.											
1	4	+21.6	33.94	0.963	35.3	660			68.5		
2	4	+23.9	35.90	0.620	57.9	451			72.7		
3	3	+27.7	38.18	0.814	46.9	670			82.3		

daily elimination of 0.145 gm. of S during 24 days of fasting as the basal sulfur metabolism of this dog, there was only 50 per cent increase during the 11 days of refeeding, or actually 0.218 gm. of S, whereas the daily intake was on the average about 0.873 gm. We observed, therefore, in this dog a sparing of the sulfur during fasting and a greedy retention during a period of intensive anabolism.

Unfortunately the sulfur partition in the urine was not carried out completely, only the total S and the inorganic sulfate having

been determined. It will be noted that the inorganic sulfate in this dog constitutes a very large part of the total sulfur eliminated, about 85 per cent. This fraction diminished during the course of the fast, especially during the second half, to about 71 per cent. During refeeding there is an obvious tendency to rise, the inorganic sulfate rapidly increasing to 82.3 per cent on the 9th to 11th refeeding day, which is practically the same proportion which has been found in the prefasting period.

TABLE III.

Dog 7, female collie; 16.08 kilos.

Period.	Duration.	Change in weight.	Total N.	Total S.	N:S ratio.	S.			Per cent of total S.		
						Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.
	days	*per cent*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*			
Preliminary.											
1	3		32.85	2.136	15.4	1603	237	296	75.0	11.1	13.9
2	4		49.10	2.868	17.1	2214	280	374	77.2	9.8	13.0
Fasting.											
1	3	−5.5	11.79	0.660	17.9	600	20	40	90.1	3.3	6.6
2	3	−9.8	10.53	0.680	15.5	542	97	41	79.7	14.3	6.0
3	4	−14.9	13.06	0.707	18.4	608	46	53	86.0	6.5	7.5
4	4	−20.7	12.95	0.722	17.9	627	53	42	86.8	7.3	5.9
5	4	−24.4	12.23	0.672	18.2	541	60	71	80.5	8.9	10.6
6	4	−28.8	11.34	0.561	20.2	488	54	19	87.0	9.6	3.4
7	4	−32.8	11.66	0.584	19.2	502	34	48	86.0	5.8	8.2
8	4	−37.7	11.65	0.541	21.5	464	37	40	85.6	6.9	7.4
9	3	−40.4	4.32	0.344	12.5	271	38	35	78.8	11.1	10.1

Dog 7 (Table III) was fed a diet in which both N and S were directly determined by analysis and were found to be in a ratio of 21.6:1. During the preliminary period which lasted 7 days, the dog had a small positive nitrogen balance, or was actually in nitrogen equilibrium, when proper allowance is made for the nitrogen lost in the feces. Nevertheless, there was a definite loss of sulfur, the excretion of S in the urine exceeding the intake for 7 days by 0.630 gm. The N:S ratio in the urine is low (16.3). The dog fasted 33 days without water until it had sustained a loss of over 40 per cent in weight. The N:S ratio changes very little

during the first half of the fast but in the last half it shows a tendency to increase somewhat.

The sulfur partition in the urine presents certain interesting points. About 76 per cent of the sulfur was eliminated in the form of inorganic sulfates during the preliminary period but this fraction immediately increased to much higher values with the beginning of the fast and the average for the entire fasting experiment was 84.5 per cent of the total S. This rise in the inorganic sulfate fraction is partly due to a diminution of the ethereal sulfate but more particularly of the neutral sulfur fraction.

This animal showed signs of ailment during the seventh fasting period; towards the end of the eighth period it was definitely sick and died during the 4th day of the ninth fasting period. The urine for the last few days showed the presence of albumin in increasing amounts. The condition was diagnosed by Dr. O. F. Reihart as distemper. During this last fasting period the nitrogen excretion was much more affected than that of the sulfur, resulting in an unusually low N:S ratio. It is worth noting that in spite of this severe infection during the last few days of the experiment the urinary sulfur partition had not been much altered except during the last 3 days when the dog became markedly toxemic. During these last days the ethereal and neutral sulfur have both increased but not beyond the original level found in the prefasting period.

Dog 11 (Table IV) had been practically in nitrogen equilibrium during the preliminary periods (7 days) but like Dog 7 the sulfur excretion exceeded the intake by 0.812 or 0.116 gm. per day. The average N:S ratio in the urine was 17.5, whereas that of the food consumed was 21.6. During the entire fast lasting 37 days, during which the dog lost 31.2 per cent in weight, the urinary N:S ratio varied within narrow limits. The average ratio was 18.3, which is not materially different from the ratio during the prefasting periods. In the subsequent 12 days of refeeding about 5 gm. more of sulfur were retained than was enough to make good all the sulfur lost during the fast and to provide for the basal sulfur metabolism. This is especially striking when we consider that the nitrogen retention was hardly sufficient to cover 40 per cent of the nitrogen lost during the fast. The urinary N:S ratio was greater (20) than during either the preliminary or the fasting

periods, showing a remarkable degree of stability in this animal. If all the N and S retained during the three refeeding periods had been utilized in protein synthesis, the new protein would contain more than twice the usual proportion of sulfur.

The sulfur partition in the urine reveals a high proportion of inorganic sulfate (75.5 per cent) and a low ethereal sulfate (3.5 per cent). In the course of the fast the ethereal sulfate fraction

TABLE IV.

Dog 11, female mongrel; 20.0 kilos.

Period.	Duration.	Change in weight.	Total N.	Total S.	N:S ratio.	S.			Per cent of total S.		
						Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.
	days	*per cent*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*			
Preliminary.											
1	4		39.86	2.535	15.7	1923	102	510	75.9	3.9	20.2
2	3		29.52	1.527	19.3	1131	47	349	74.1	3.1	22.8
Fasting.											
1	4	−5.6	18.60	0.998	18.6	680	58	260	68.2	5.8	26.0
2	4	−9.7	14.46	0.752	19.2	538	72	142	71.5	9.6	18.9
3	4	−12.6	12.31	0.723	17.0	476	69	178	65.8	9.5	24.7
4	4	−15.7	10.95	0.600	18.3	382	55	163	63.8	9.1	27.1
5	4	−18.0	10.32	0.555	18.6	410	60	85	73.9	10.8	15.3
6	4	−22.0	9.84	0.530	18.5	362	34	134	68.3	6.4	25.3
7	4	−26.7	10.02	0.509	19.7	383	58	68	75.3	11.4	13.3
8	4	−29.5	9.82	0.541	18.2	409	49	83	75.6	9.0	15.4
9	4	−31.2	9.34	0.559	16.7	376	56	127	67.3	10.0	22.7
Refeeding.											
1	4	+4.8	28.69	1.368	20.9	960	42	364	70.1	3.3	26.6
2	4	+13.8	34.48	1.814	19.0	1340	0	474	73.8	0	26.2
3	4	+22.2	44.24	2.086	20.3	1445	42	599	69.3	2.0	28.7

increases up to 11.4 per cent, while the inorganic sulfates and the neutral sulfur undergo irregular but not seemingly significant changes. The point of special interest, however, is the sharp drop in the ethereal sulfates during refeeding, although the food intake, at any rate during the last two refeeding periods, was about 30 per cent more than in the preliminary. In spite of this increase in the digestive activity the ethereal sulfate fraction was quickly

reduced to the prefasting proportion. The neutral sulfur fraction was increased.

Dog 6 (Table V) had a positive nitrogen and sulfur balance during the preliminary periods, and the two were retained practically in the same ratio (20.9) as was found in the diet (21.6). During the first 10 days of fasting the urinary N:S ratio increased very markedly (average 31.5), indicating a retention of sulfur, but during the remaining part of the fast the ratio remained practically as during the preliminary period.

The fast was followed by two refeeding periods of 3 days each, during which the dog retained only 0.47 gm. of sulfur or about 12 per cent of the amount lost for the entire fast. The nitrogen retention was relatively twice as great, and more than 20 per cent of the nitrogen lost was thus made up. The situation is, therefore, exactly the reverse of what was found in Dog 11. The urinary N:S ratio during the refeeding periods diminished somewhat. Had all the nitrogen and sulfur retained been utilized in the synthesis of tissue protein this new protein would have about one-third as much sulfur as usual.

The sulfur partition in the urine of this dog reveals the singular fact that the inorganic sulfate fraction is unusually low, being only about 50 per cent of the total sulfur eliminated, whereas the unoxidized sulfur fraction is exceptionally high (43 per cent). In the course of the fast certain changes in the sulfur partition occur with striking regularity. The ethereal sulfates remain constant at first, then increase to about double the proportion, and diminish very greatly during the last two periods. On refeeding, this fraction remains still as low as it was toward the end of the inanition experiment. The neutral sulfur fraction diminished very abruptly as soon as the dog commenced to fast, but gradually began to rise again and increased from 11.4 per cent in the first fasting period, to 37.3 per cent of the total sulfur, in the last period. The inorganic sulfate fraction, on the contrary, rising suddenly to almost 83 per cent of the total sulfur excretion at the beginning of the inanition decreases gradually and remains practically constant at about 60 per cent. The changes in both fractions, the inorganic and neutral sulfur, are thus definitely reciprocal. The more remarkable fact, however, is the rapidity with which the peculiar sulfur partition in the urine of this dog

is reestablished when it is fed again. The inorganic sulfates undergo a marked diminution and form again 50 per cent of the total S, while the neutral sulfur sharply increases and by the end of 6 days of refeeding is nearly at the original level noted in the prefasting period. This readjustment in the sulfur partition suggests strongly that we are dealing with a metabolic condition peculiar to this dog.

TABLE V.

Dog 6, female Boston terrier; 13.03 kilos.

Period.	Duration.	Change in weight.	Total N.	Total S.	N:S ratio.	S.			Per cent of total S.		
						Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.
	days	*per cent*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*			
Preliminary.											
1	2		18.76	0.918	20.4	458	63	397	49.9	6.9	43.2
2	4		38.28	1.660	23.0	852	99	709	51.3	6.0	42.7
Fasting.											
1	3	−7.1	10.91	0.323	33.8	267	19	37	82.6	6.0	11.4
2	3	−12.5	10.53	0.373	28.2	289	25	59	77.6	6.6	15.8
3	4	−17.3	13.26	0.407	32.6	258	27	122	63.5	6.6	29.9
4	4	−21.0	11.57	0.486	23.8	315	40	131	64.8	8.2	27.0
5	4*										
6	4	−28.0	10.54	0.495	21.3	297	42	156	60.0	8.5	31.5
7	4	−31.7	10.31	0.512	20.1	293	60	159	52.2	11.7	36.1
8	4	−34.4	10.18	0.469	21.7	285	18	166	60.7	3.8	35.5
9	4	−37.6	9.65	0.491	19.7	302	6	183	61.6	1.1	37.3
Refeeding.											
1	3	+10.8	24.40	1.330	18.3	519	43	768	39.0	3.3	57.7
2	3	+18.7	24.48	1.323	18.5	665	22	636	50.2	1.8	48.0

* Attack of cystitis; urine for this period discarded.

Dog 10 (Table VI) had been losing daily 0.054 gm. of sulfur during the preliminary period, although there was probably a small positive nitrogen balance at the same time. The urinary N:S ratio was practically 16, which is considerably lower than that in the food (21.6). In the course of a long fast lasting 36 days there has been very little variation in this ratio, which was on the average for the entire inanition experiment 15.3. During the subsequent 12 days of refeeding the N:S ratio increased to 20.8

(average), which is in good agreement with the N:S ratio of the diet (20.6). The nitrogen and sulfur during the refeeding periods were retained in about the same proportion, about 43 per cent of the loss in sulfur and 54 per cent in nitrogen having been repaired.

The sulfur partition in the urine presents certain interesting points. The inorganic sulfate fraction is 69 per cent (average) and the neutral sulfur fraction is relatively large (30.4 per cent),

TABLE VI.

Dog 10, female Boston terrier; 15.7 kilos.

Period.	Duration.	Change in weight.	Total N.	Total S.	N:S ratio.	S.			Per cent of total S.		
						Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.
	days	*per cent*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*			
Preliminary.											
1	4		29.51	1.795	16.4	1163	3	629	64.8	0.2	35.0
2	3		20.88	1.345	15.5	986	12	347	73.2	1.0	25.8
Fasting.											
1	4	−5.3	15.32	0.958	16.0	707	75	176	73.8	7.8	18.3
2	4	−9.3	10.90	0.688	15.8	505	64	119	73.4	9.3	17.3
3	4	−12.2	9.74	0.666	14.6	498	52	116	74.8	7.8	17.4
4	4	−15.2	8.97	0.624	14.4	459	71	94	73.6	11.3	15.1
5	4	−18.4	10.11	0.628	16.1	496	58	74	79.0	9.2	11.8
6	4	−22.0	7.20	0.487	14.8	407	18	62	83.5	3.8	12.7
7	4	−28.4	8.70	0.425	20.5	321	34	70	75.5	8.0	16.5
8	4	−32.0	7.46	0.509	14.7	394	26	89	77.4	5.1	13.5
9	4	−35.0	8.34	0.572	14.6	408	24	140	71.3	4.2	24.5
Refeeding.											
1	4	+9.4	17.60	0.833	21.1	618	60	55	74.2	7.2	18.6
2	4	+18.0	24.73	1.288	19.2	1028	0	320	79.9	0	20.1
3	4	+24.9	29.00	1.306	22.2	892	106	308	68.4	8.1	23.5

while the ethereal sulfates are very low (0.6 per cent). With the onset of inanition there is a sudden very marked increase in the ethereal sulfate fraction (both absolute and relative) with a tendency, however, to diminution in the advanced periods of the fast. The series of changes is not unlike that observed in Dog 6 except that in the latter during the preliminary period the ethereal sulfate fraction was larger. The neutral sulfur fraction diminishes

gradually during the inanition except for a big increase occurring in the last fasting period, while the inorganic sulfate fraction increases up to about the middle of the fast and then diminishes again. The average proportion of this fraction is 75.8 per cent of the total sulfur excretion. On refeeding the neutral sulfur tends to increase and the inorganic sulfate to decrease, approaching the prefasting level. The ethereal sulfate fraction for the three refeeding periods is neither absolutely nor relatively greater than during the inanition experiment though considerably larger than in the preliminary period. The behavior of the neutral

TABLE VII.

Dog 9, female bulldog; 16.4 kilos.

Period.	Duration.	Change in weight.	Total N.	Total S.	N:S ratio.	S.			Per cent of total S.		
						Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.
	days	*per cent*	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*			
Preliminary.											
1	4		48.00	2.660	18.0	2018	32	610	75.9	1.2	22.9
2	4		54.40	2.920	18.6	2370	68	482	81.2	2.3	16.5
Fasting.											
1	3	−5.7	12.98	0.972	13.4	674	0	298	69.4	0	30.6
2	4	−11.8	16.13	1.100	14.7	794	20	286	72.2	1.8	26.0
3	4	−18.0	17.60	1.202	14.7	945	0	257	78.5	0	21.5
4	4	−23.2	21.05	1.156	18.2	960	5	191	83.0	0.5	16.5
5	4	−29.3	21.20	1.183	17.9	936	38	209	79.0	3.3	17.7
6	3	−33.2	13.56	0.808	16.8	584	66	158	72.3	8.2	19.5

sulfur fraction in Dogs 6 and 10 would be difficult to interpret on the assumption that it rises and falls with the changes in intestinal putrefaction, being much larger during the inanition experiment than during the prefasting or refeeding periods in spite of the large amounts of food then consumed.

Dog 9 (Table VII) had a urinary N:S ratio of 18.3 during the preliminary period. The N:S ratio of the food was 21.6. With the commencement of the fast the urinary N:S ratio dropped to about 14 (13.4 to 14.7) but increased to practically the prefasting value in the remaining three experimental periods (17.6). The

last part of the experiment, however, is complicated by the circumstance that the dog developed distemper. For the last week the dog ran a high temperature, showed increasing amounts of albumin in the urine, and died before the 6th fasting period was completed.

A study of the urinary sulfur partition shows a remarkably low ethereal sulfate which during the fast becomes even smaller and in some periods disappears entirely. Only in the last period when the dog's condition was really very critical did the ethereal sulfate fraction increase appreciably. In this respect the results are similar to those noted in Dog 7 which also died from distemper. The inorganic sulfate fraction which was rather large (78.5 per cent) during the preliminary period diminished at first when the dog began to fast, but increased gradually, and then during the last two fasting periods declined again. The changes in the neutral sulfur are practically reciprocal to those in the inorganic sulfate.

DISCUSSION AND SUMMARY.

From a survey of the results of the experimental study it is obvious that in dogs on a fairly uniform diet the urinary N:S ratio varies within wide limits (16.1 to 29.7). During protracted fasting the variations in the N:S ratio likewise range from 15.3 to 27.5, and, with the exception of Dog 2, there is a clear tendency for the ratios to be of the same order of magnitude whether the animal is fed or fasted. The urinary N:S ratio may under some conditions be determined by the nature of the food consumed. Thus, Fay and Mendel (7) have shown that when egg white is fed to dogs as the sole source of protein the N:S ratio in the urine is practically the same as in the food. It is questionable, however, whether Siven's idea (14) of the correspondence between the food and urinary N:S ratio can generally be subscribed to. Fay and Mendel's experiments fail to corroborate this hypothesis, nor do our results give it general support. We did find that the two ratios were very nearly the same in Dog 6 during the preliminary period and in Dog 10 during the refeeding periods, but this seems more a coincidence than a general phenomenon.

Wilson (16) has shown that sulfur is more effectively spared during metabolism than nitrogen by fat and carbohydrate in the

diet, which suggests that the N:S ratio of the end-products discarded through the urine merely reflects a complex metabolic response of the organism.

During fasting the N:S ratio in the urine of our dogs shows considerable stability. In five out of seven animals there has been little of unusual interest in this respect. However, in Dog 5, the N:S ratio increased greatly during fasting and, subsequently, upon refeeding the rise in the ratio became quite extraordinary, associated with an extensive sulfur retention. In Dog 6 we likewise observed a great increase in the urinary N:S ratio but this occurred only in the early part of the fast. In the more advanced phases of the fast and later during the refeeding periods the N:S ratio showed no unusual changes. Carpenter (2) in his studies of the urine from fasting steers reports values for nitrogen and sulfur elimination. On recalculation these results betray an extreme degree of variability in the urinary N:S ratios from day to day (Steer C, 9.0 to 74.0; Steer D, 6.9 to 57.2) which, unless they can be accounted for by some analytical condition, are most extraordinary.

We must conclude from an extensive study of the N:S ratio that this is an expression of such complex metabolic relationships as to make any idea of judging by it the nature of tissue undergoing catabolism during fasting fatuous. The most significant point, it seems, is that the relative excretion of nitrogen and sulfur in the urine is an individual metabolic response. Some dogs show high and others low N:S ratios, and this fact must be emphasized even though we cannot offer any explanation for it now.

Does the sulfur in the urine represent protein catabolism? It is difficult to conceive of protein undergoing partial hydrolysis with its sulfur-free and sulfur-containing moieties meeting with different fates. It is very probable that cysteine accounts for only a small part of the sulfur content of protein, the nature of the other sulfur compound still being unknown. At the same time, the urinary N:S consistently fails to conform to any definite protein ratio. On the contrary, Mitchell and his coworkers (12) have shown that the non-protein N:non-protein S ratio in the tissue of rats on a normal and on a protein-free ration is practically the same, this sulfur being chiefly in the unoxidized form. This corresponds well with the findings of Denis and Reed (5) that in

the blood about one-half of the total sulfur present is in the unoxidized form, this distribution of the sulfur fractions not being affected by the sulfur and nitrogen level in the diet or by the rate of their excretion. This constancy of the tissue condition is undoubtedly of great physiological significance and the endogenous metabolism may be regulated to maintain this physiological level. Mitchell (12) suggests that we should not consider protein metabolism at all, but only the metabolic transformation of the non-protein nitrogenous components of the tissues. This view-point may perhaps bring us nearer to an appreciation of the sulfur metabolism than the futile attempt to correlate the N:S ratio of protein and of urine. The sulfur partition presents considerable interest. It was shown by Folin (8) that the per cent of total excreted sulfur which is in the form of inorganic sulfate may increase to about 90 per cent on a protein-rich diet or drops to about 60 per cent on a protein-poor diet. However, in Folin's experiments the difference in protein intake is very great, the nitrogen excretion diminishing from 16.8 to 3.6 gm. The great variation in the per cent of sulfate is due to the fact that both the ethereal and neutral sulfur fractions are practically unaffected under these dietary conditions. It seems, therefore, that all the exogenous protein metabolism results in the formation of inorganic sulfates. In our dogs, with a fairly constant protein intake, the per cent of inorganic sulfate in the urine varies nevertheless from 50.6 to 84.8 per cent. This, however, is not an unusual variation. Wolf and Oesterberg (17) found in three dogs that the inorganic sulfate constituted 52.1, 68.4, and 83 per cent of the total sulfur excretion. We calculated the results reported by Denis and Reed (6) on the sulfur partition in the urine of their dogs and found that the inorganic sulfate there also forms 52 to 74 per cent of the total sulfur excretion. These dogs did not receive apparently as uniform a diet as in our experiments. Unfortunately Denis and Reed give no information on the nitrogen excretion. Their dogs were fed either the Cowgill mixture, meat, or bread and milk, and the highest sulfate per cent was found in dogs on the milk and bread while the lowest per cent in dogs was on the meat diet.

We have examined the various factors which might be held responsible for the variation in the sulfate fraction but failed to discover any connection between this and the amount of water

consumed or voided, or the absolute amount of sulfur and nitrogen excreted. Nevertheless, the sulfur partition seems to be peculiar to each dog and when the animals are fed following an intervening prolonged fast there is a definite tendency for the reestablishment of the prefasting sulfur partition. We did find, however, that there is some relationship between the type of dog experimented on and its sulfur partition. Thus, we found from incidental protocol notes the following description of the experimental animals, which we reproduce with the corresponding sulfur partitions.

Dog No.	Inorganic S. *per cent*	Neutral S. *per cent*
5 Collie	84.8	
2 "	77.9	17.7
7 "	76.1	13.4
9 Bulldog	78.5	19.7
11 Mongrel	75.0	21.5
10 Boston terrier	69.0	30.4
6 Terrier type*	50.6	43.0

*Hele (18) in a study of the synthesis of ethereal sulfates in a terrier records data on the sulfur partition in the urine from which the inorganic fraction can be calculated as 60 to 64 per cent of the total. These values represent the averages from a number of determinations during the preliminary periods of two separate experiments performed about 2 years apart. This last fact makes the similarity in the results the more striking.

It would be hazardous to speculate from so few observations, especially since the breed of three of our dogs (Nos. 6, 9, 11) is not as definite as in the remaining ones, but it is striking that all the three collies showed a high inorganic sulfate proportion, while the terriers, on the contrary, showed a very low proportion. What has been said of the inorganic sulfates holds equally for the neutral sulfur except that this fraction behaves in a reciprocal manner to the inorganic sulfate. In view of the fact that our results have already indicated very strongly the individual peculiarity in the matter of the sulfur partition, we would suggest, at least tentatively, that the sulfur metabolism varies in different breeds of dogs independently of the diet. We know, in the case of uric acid metabolism, that the Dalmatian dog is different from other breeds of dogs, and furthermore that upon hybridization of the Dalmatian the uric acid metabolism of the offspring

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shows every gradation between the two extremes depending upon the hereditary make-up of the hybrid. May we not, likewise, be dealing with a racial peculiarity in the sulfur metabolism?

Regarding the ethereal sulfate fraction, we know that part of it is derived from the conjugation with bacterial putrefaction products. However, it is also generally recognized that part is probably of endogenous origin. In our experimental animals during fasting we found three types of response: the ethereal sulfate fraction increased appreciably during the fasting period (Dogs 10, 11, 2) or remained unchanged at least for a time (Dogs 6, 7) and, finally, greatly decreased even to the vanishing point (Dog 9). On the theory that the ethereal sulfates are associated with a conjugation of putrefactive products it would be difficult to explain the great increase (both relative and absolute) in this fraction in the urine of most of our dogs, and especially its almost complete disappearance in Dog 9.

The behavior of the neutral sulfur fraction has been already fully discussed in the detailed description of the results pertaining to each dog. In two cases (Dogs 7, 10) there was a definite reduction, in two cases (Dogs 2, 11) an increase, while in two other cases there was a sharp initial diminution followed by a gradual rise (Dog 6) or an initial rise followed by a drop (Dog 9). It is impossible, therefore, to say that fasting is necessarily associated with a rise in this unoxidized sulfur moiety or to deduce from this the hypothesis that the oxidative processes are impaired during inanition (13).

CONCLUSIONS.

1. The average urinary N:S ratio in dogs on a fairly uniform diet ranges from 16.1 to 29.7. During prolonged fasting these dogs (with only one exception) show the same average ratios (15.3 to 27.5). The N:S ratio is, therefore, peculiar to the individual dogs, some having high and others low ratios.

2. The experimental evidence fails to demonstrate a relationship between the N:S ratio of the food and of the urine.

3. It is impossible to determine from the urinary N:S ratios the nature of the tissue undergoing catabolism.

4. The variations in ethereal sulfate during fasting suggest that this sulfur fraction must be chiefly of metabolic origin.

5. The neutral sulfur fraction is subject to very irregular changes, but the evidence does not permit the conclusion that these are associated with diminished oxidative processes in the fasting organism.

6. The variations in the inorganic sulfate fraction are likewise irregular. This fraction is influenced more by the individuality of the dog than by the diet, some dogs voiding a urine with a high per cent of inorganic sulfate and low neutral sulfur, while other dogs show the reverse condition.

7. Upon refeeding, the inorganic sulfates tend to assume the same proportion as before the fast, independently of the changes which this sulfur fraction had undergone in the course of inanition.

BIBLIOGRAPHY.

1. Bendict, F. G., *Carnegie Institution of Washington, Pub. No. 203*, 1915.
2. Carpenter, T. M., *Am. J. Physiol.*, 1927, lxxxi, 519.
3. Cathcart, E. P., *Biochem. Z.*, 1907, vi, 109.
4. Cowgill, G. R., *J. Biol. Chem.*, 1923, lvi, 725.
5. Denis, W., and Reed, L., *Am. J. Physiol.*, 1927, lxxxiii, 47.
6. Denis, W., and Reed, L., *J. Biol. Chem.*, 1927, lxxiii, 51.
7. Fay, M., and Mendel, L. B., *Am. J. Physiol.*, 1925, lxxv, 308.
8. Folin, O., *Am. J. Physiol.*, 1905, xiii, 99.
9. Halpern, M., *Biochem. Z.*, 1908, xiv, 134.
10. Kahn, M., and Goodridge, F. G., Sulfur metabolism, Philadelphia, 1926.
11. Lewis, H. B., *J. Biol. Chem.*, 1916, xxvi, 61.
12. Mitchell, H. H., Nevens, W. B., and Kendall, F. E., *J. Biol. Chem.*, 1922, lii, 417.
13. Morgulis, S., Fasting and undernutrition, New York, 1923.
14. Siven, V. O., *Skand. Arch. Physiol.*, 1901, xi, 308.
15. von Wendt, G., *Skand. Arch. Physiol.*, 1905, xvii, 211.
16. Wilson, H. E. C., *Biochem. J.*, 1925, xix, 322.
17. Wolf, C. G. L., and Oesterberg, E., *Biochem. Z.*, 1911, xxxv, 329.
18. Hele, T. S., *Biochem. J.*, 1924, xviii, 110.

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A NOTE ON THE KRAMER-TISDALL POTASSIUM METHOD.

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In the determination of potassium in blood by the Kramer-Tisdall method (1, 2) as the complex potassium sodium cobaltinitrite, it is assumed that the reaction takes place according to the equation: $Na_6(Co_2(NO_2)_{12}) \cdot H_2O + 4\ KCl = 2K_2Na(Co(NO_2)_6) \cdot H_2O + 4NaCl$. The potassium is then determined from the titration of the precipitate with 0.02 N $KMnO_4$. Kramer and Tisdall give the factor 0.071 for the conversion of cc. of 0.01 N $KMnO_4$ to corresponding quantities of potassium. This factor, however, is not derived stoichiometrically and is a purely empirical value. We found that it is by no means constant for different reagents and have been redetermining it from analyses on known quantities (3). As a matter of fact, after a long study of the method, we came to the conclusion that there is no fixed factor for converting the permanganate volume into mg. of potassium, but that this varies according to the amount of potassium analyzed. The titration results when plotted as ordinates against mg. of K as abscissæ give a straight line. We recommend, therefore, that each new reagent should be standardized with varying amounts of a known potassium solution. In analyses of unknown quantities, the mg. K can now be read off directly from the curve, thus dispensing entirely with the complicated calculation and avoiding the error resulting from the use of a fixed factor. In Fig. 1 we reproduce several curves. The heavy line is a theoretical curve, a fixed factor of 0.071 being assumed. The lighter lines are all experimental curves obtained from analyses of a standard solution of KCl. It will be seen that the experimental curves are also straight lines but with a *different slope*. They intersect the

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theoretical curve but the point of intersection is not the same for different reagents. Thus with one reagent the intersection would

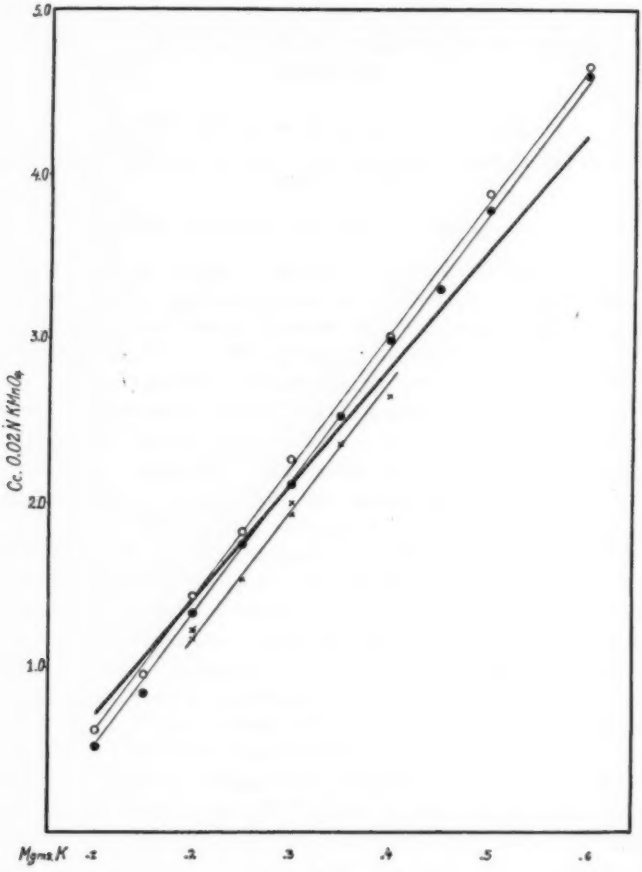

FIG. 1.

occur between 0.5 and 0.6 mg. of K, while with another sample the intersection is between 0.2 and 0.3 mg. of K. It is also obvious why a factor of 0.071 was selected if the determinations were

confined entirely to the range of 0.2 to 0.3 mg. of K, as is usually the case in blood plasma analyses. In view of the fact that P is supposed to interfere with the potassium determination, it may be of interest to mention that we obtained identical curves with both KCl and KH_2PO_4 standard solutions, so that P, at any rate in an equivalent concentration, does not seem to affect the results of the potassium analysis.

Considering that the experimental curves are straight lines, one can calculate the results from the usual intercept form of the equation for a straight line, $\frac{X}{a} + \frac{Y}{b} = 1$, where a and b are the X and Y intercepts, respectively. With the aid of this equation the quantities of K can be calculated beyond the limits of the plot. Thus, for the experimental curve marked by solid dots the equation becomes: $0.44\ X - 0.0516\ Y = 0.0213$, where X = mg. of K and Y = cc. of 0.02 N $KMnO_4$.

BIBLIOGRAPHY.

1. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlviii, 223.
2. Kramer, B., *J. Biol. Chem.*, 1920, xli, 263.
3. Morgulis, S., *Am. J. Physiol.*, 1928, lxxxiv, 350.

THE EFFECTS OF INADEQUATE VITAMIN A ON THE SEXUAL PHYSIOLOGY OF THE FEMALE.*

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In 1922 we noted a new and characteristic test for deficiency in fat-soluble vitamin A—the constant appearance of cornified cells either predominantly or exclusively in the vaginal smear.[1] In the intervening time we have conducted many thousands of tests with dietary mixtures low or lacking in vitamin A and have never seen the test fail. It is earlier and, since invariable, more constant in its appearance than xerophthalmia or abscesses of the glands at the base of the tongue and while perhaps not earlier than growth decline, is pathonomonic of vitamin A deficiency as growth decline is not. The test is hence invaluable in all studies of reproduction or other body functions in which we must be assured of conditions during a time of vitamin A delinquency. The constant appearance of cornified cells[2] in the vaginal smear

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[1] Evans, H. M., and Bishop, K. S., On an invariable and characteristic disturbance of the reproductive function in animals reared on a diet poor in fat soluble vitamine A, *Anat. Rec.*, 1922, xxiii, 17.

[2] Since these cells normally occur during the epoch of active heat, or estrus, it might be conceived that the prolonged appearance of cornified cells indicates prolonged estrus. This conception is invalidated both by the behavior of animals on diets low in vitamin A and by the condition of the ovaries of these animals. Such animals are not in continuous estrus as shown by daily tests, or better, by the constant presence of active males in their cage. Furthermore, the ovaries of animals showing this test for vitamin A delinquency do not exhibit ripe follicles or vesicles characteristic of the estrous stage and, what is more important, the ovary's lack of mediation in this sign is conclusively shown by the exhibition of the sign after double ovariectomy.

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indicates impoverishment in vitamin A during the time such tests are conducted. The introduction of a high source of vitamin A (cod liver oil, 1 to 3 drops daily) may change the vaginal smear to the normal one of diestrus in 24 hours and invariably accomplishes this within 3 days. That the change is due to the introduced vitamin A rather than the antirachitic vitamin D seems conclusively shown by the same continuous exhibition of the sign (prolonged cornified cells) in experiments which we have recently conducted with Diet 251, in which the lard was irradiated with a Hanovia lamp.

Diet 251.		*Diet 56.*	
Casein (extracted)	25	Casein (extracted)	18
Corn-starch (cooked)	69	Corn-starch (cooked)	54
Lard (irradiated)	2	Lard	24
Salts	4	Salts	4
Yeast....0.5 to 0.7 gm. daily.		Yeast....0.5 to 0.7 gm. daily.	

Twelve female rats were reared from the day of weaning (21st day of life) on Diet 56, with 0.5 to 0.7 gm. of whole dried yeast daily. When the animals were 60 days of age there were added daily to the yeast 6 drops of wheat germ oil to assure an adequate level of vitamin E. The average age at the occurrence of the first estrus was 64 days (36 to 101). Slightly after the 100th day of life a normal, sexually active male was placed nightly in the cage of each female. Search was made each morning for the copulatory plug or residual spermatozoa. The method, we believe from an extensive experience, is a practically infallible one to detect copulation. The animals were studied in this way until approximately the completion of the 200th day of life. 22 per cent of the copulations resulted in the birth of living young $\left(\frac{13}{59}\right)$. It is interesting that in these cases there was a continuous exhibition of the sign of vitamin A delinquency (continuous cornified cells) throughout gestation, showing that whatever other delinquencies existed in our diet (and we confessedly did not take pains to insure an adequate level of vitamin D) the animals suffered from impoverishment in vitamin A and yet in the presence of the vitamin E were able in somewhat less than one-fourth of the instances to complete gestation. The large majority of copulations did not show the implantation sign. We are hence

sure that resorption (characteristic of vitamin E delinquency) did not take place in these cases but that failure must have occurred in some phase of the reproductive mechanism anterior to implantation. Sections of the oviduct of such cases (from 1 to 3 days after the finding of spermatozoa) show degenerated eggs in the last portion of the tube. If fertilization occurred, death must have ensued shortly thereafter, for there were no indications of blastogenesis. There is the highest probability from these microscopic findings that fertilization did not occur, although proof that the eggs were shed is given. It is clear, then, that the majority of the eggs, although coming from follicles which secreted the estrus hormone and inducing mating in their possessors, were either too infirm to be fertilized or the spermatozoa were incapable of reaching them. It is highly interesting that in the case of every individual of the group at least one successful gestation was accomplished at some time in its history, not invariably in the earliest part of it. The variation may be due to differences in the viability of the various crops of eggs, or to altered conditions in the reproductive tract which would impede or inhibit the journey of the spermatozoa, a condition quite possible since all the epithelia are changed in animals on low vitamin A diets. When litters were born they were not greatly under the expected size and weight. Many of the histories showed copulations at 5 day intervals, indicating rhythmic estrus and ovulation, a rhythmicity which we could not have detected by the vaginal smear on account of the continuous exhibition of the cornified sign. The rhythmic coitus, if it may be so styled, further demonstrates that the condition of pseudopregnancy is not induced in these animals. This contributes the further fact of impaired or inadequate internal secretions in these females.

SUMMARY.

1. Inadequate vitamin A injures the female reproductive system so that fertilization and implantation often fail. In this respect it differs radically from the reproductive impairment due to low vitamin E content, where, typically, the eggs are always healthy and implantation takes place but resorption follows.

2. A level of inadequate vitamin A can be secured, denoted by

continuous cornified cell vaginal smears, during which estrus and ovulation occur and are, in fact, fairly frequent. This is only demonstrable by the continuous presence of the male for no changes occur in the vaginal cell types. Four-fifths of the copulations eventuate in failed implantation. A great many of them fail even to establish the condition of pseudopregnancy since another estrus occurs in 5 days. About one-fifth of the copulations leads to the birth of litters. It is interesting that throughout gestation the cornified cell smear continues. Normally the vaginal epithelium in pregnancy is high columnar in type.

REDUCING SUBSTANCES IN THE BLOOD OF THE DOG-FISH, SQUALUS SUCKLII, AND CERTAIN OTHER FISHES.*

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Though little is known of the nature or function of the reducing substances present in the blood of fishes, there is nothing to suggest that they differ from those in mammalian blood. Since the development of the Folin-Wu technique, values have been recorded which are quite analogous to the sugar values normally obtained from the blood of the higher vertebrates, except that figures showing very wide variations have been obtained, even in fish of the same species. The cause of these variations is still obscure. Hyperglycemia induced by asphyxiation has been generally accepted as one of the underlying factors, but there is some evidence (Menten (1)) that the amount of sugar in the blood depends upon the quantity of food ingested and the time of feeding. This is a point which seems to have been overlooked by most investigators, who, relying probably upon the fact that the food is largely protein in character, have usually endeavored to take the blood sample as soon as possible after catching the fish. Since the diet contains little or no carbohydrate, the blood sugar is in all probability at least in part derived from protein, and the possibility of a certain amount of alimentary hyperglycemia cannot be ignored. The primary object of this investigation was to obtain the minimal normal values for reducing substances in the blood, hence as a routine procedure before samples were taken, the fish were kept for at least 2 days under as nearly as possible normal conditions, but without food.

* A study from the Stations of the Biological Board of Canada.

All the fish used in this investigation were obtained either in nets or by means of a trawl, with the exceptions of the dogfish, caught on a set line, and the lampreys, caught by hand. As soon as possible after capture, they were transferred to a large wooden tank whose sides were latticed to allow passage of sea water; this tank was kept attached to a floating raft and immersed in the sea. When required, a fish was transferred with a landing net to the raft, where the sample was usually obtained by severing the caudal vessels and allowing the blood to flow into a tube whose sides had been rinsed with potassium oxalate solution and then drained. This was found by experience to be the quickest and most satisfactory method, a period of 2 minutes being more than ample for the whole procedure. Such rapidity is of some importance, since Scott (2) claims to have observed in the case of the dogfish, *Mustelis canis*, a pronounced asphyxial hyperglycemia which attained its maximum value within 4 minutes of the removal of the fish from the sea. In the case of the lampreys blood was obtained by severing the third branchial cleft, but some trouble was at first experienced owing to rapid clotting. This was overcome by wiping the surface of the skin with a cloth moistened with potassium oxalate solution before making the incision.

In the preparation of the blood filtrates the ordinary Folin-Wu technique was adhered to, but was slightly modified for dogfish blood since its cell volume is relatively much less than that of teleosts and mammals. In this case, the blood was laked with 5 volumes of water, while 0.5 volume of each of the precipitating reagents was found to be adequate.

It should be mentioned that the minimum dilution necessary for dogfish blood was found to be 5 volumes of water to 1 of blood, microscopic examination showing clearly the presence of corpuscles in bloods of less dilution. Menten diluted 2 cc. of blood with 5 cc. of water, followed by 1.5 cc. respectively of the precipitating reagents. It is doubtful whether complete laking was obtained with these proportions, and there is no evidence that the sugar in fish blood is equally distributed between the corpuscles and the plasma.

Folin (3) and Benedict (4, 5) by the use of different reagents have shown that reduction values can be obtained which are considerably lower than those obtained with the original Folin-Wu

reagents, and are therefore presumably closer to the true glucose value. These new reagents have only been used with mammalian blood filtrates, but there seems no reason to believe they are not equally applicable to fish blood. Accordingly in the majority of the estimations which have been carried out on the blood of various fishes, all three methods have been used simultaneously on each filtrate. For further purposes of comparison, in a few cases the blood was treated according to the mercuric nitrate precipitation method used by Harned (6), which, he claims, gives a filtrate free from nitrogenous organic solutes. In order to estimate with reasonable accuracy amounts of reducing substances over a fairly wide range, three standard solutions were used containing respectively 0.1, 0.05, and 0.02 mg. of glucose per cc.

The Benedict method has the advantage of giving more deeply colored solutions which facilitate colorimetric comparison, particularly in those cases in which the sugar value is very low. This advantage is, however, offset by the color of the complex tungstic acid reagent itself, which gives quite a definite blank. Benedict states (5) that the color "is so slight that we have made no effort to improve the method in this respect. . . . When the two [unknown and standard] are diluted it will be readily seen that the color of the blank is so faint as to be entirely unobjectionable." The results which I have obtained, using Merck's purest reagents, do not entirely accord with such statements, at any rate as far as the use of dilute standards is concerned, and such dilute standards were essential for the results here recorded. The color developed in a blank test was compared with that of a solution containing 0.02 mg. of glucose per cc., and the cup measurements showed a mm. comparison of 10:21 (standard: blank). This permitted application of a mathematical correction to the results obtained by this method. The uncorrected and corrected figures are shown in Table I. That the majority of these figures do not show a greater difference is due to the use of the three standards. Obviously, the greater the difference in depth of color between the standard and the unknown, the greater will be the difference between the uncorrected and corrected figures, and consequently such a correction cannot be omitted, in cases in which the sugar concentration is low and requires weak standards.

Table I not only shows that by either the Benedict or the revised

Folin procedures lower values are obtained than by the original Folin-Wu method, but also that the Benedict figures are uniformly intermediate in value between those of the other two methods. This can only mean that, as far as these results are concerned, the Folin values more nearly represent the true glucose content of the blood, or that some more or less constant error has depressed these values below that of the true glucose content. Although the values obtained by the Harned method support the

TABLE I.

Reducing Substances Expressed in Terms of Glucose in Mg. per 100 Cc. of Blood.

Fish.	Species.	Folin.	Benedict.		Folin-Wu.	Harned.
			Uncorrected.	Corrected.		
Dogfish.	*Squalus sucklii.*	15	23	19	27	
		29	34	34	45	
		30	33	34	40	
		38	44	48	52	
		52	64	63	73	
		54	62	60	74	
		56	63	62	71	56
		64	74	74	86	
		75	85	86	99	76
Ling cod.	*Ophiodon elongatus.*	10	18	16	19	
		14	20	21	25	
		14	22	23	28	
		34	45	44	50	33

former supposition, these are too few in number to be really significant, and the possibility of an error has to be investigated.

The copper solution used by Folin in his revised method is so delicately balanced that special precautions attendant on its use have been outlined by Folin and Svedberg (7). In the analyses now reported, these have been followed in every particular. These authors further deprecate the use of too much oxalate, since excess of the anticoagulant usually results in tungstic acid being found in the blood filtrate. They state that such filtrates containing over 200 mg. per cent of sugar may give results that are

from 2 to 5 per cent too low. By the method of collecting blood necessarily adopted in this investigation, the total amount of

TABLE II.

Effect of Oxalate on the (Folin) Reduction Value of Blood.

Nature of blood.	Preparation of blood filtrate.	Oxalate used.	Reduction values (Folin).	Decrease.
		mg. per cc. blood	*mg. per 100 cc. blood*	*mg.*
Cat blood, urethane as anesthetic.	According to Folin-Wu.	1	356	
		3	348	8
		5	342	14
" " " " "	" "	1	315	
		3	310	5
		5	303	12
Dog blood, after insulin injection.	" "	1	52	
		3	50	2
		5	48	4
" " " " "	" "	1	28	
		4	27	1
Cat blood, diluted with equal volume of Ringer-Locke solution.	As for dogfish blood.	0.5	198	
		3.5	190	8
		5.5	187	11
" "	" "	0.5	183	
		2.5	177	6
		4.5	174	9
" "	" "	0.5	178	
		3.5	174	4
Dog blood, diluted with equal volume of Ringer-Locke solution.	" "	0.5	72	
		2.5	70	2
		4.5	67	5
" "	" "	0.5	13	
		2.5	13	0

oxalate used was roughly constant (the maximum being about 50 mg.), but the amount per cc. of blood varied equally of necessity

with the volume of blood obtainable. With large fish such as the dogfish and the ling cod whose weights varied from 3 to 10 pounds, and from which at least 25 cc. of blood were obtained, the amount of oxalate per cc. did not exceed 2 mg. (the original Folin-Wu procedure calls for 2 mg. per cc.), but with some of the smaller fish this concentration was undoubtedly exceeded. Consequently it seemed advisable to ascertain to what extent excess of oxalate would lower the reduction value. This part of the work was carried out subsequently when fish blood was not available. The results are shown in Table II.

TABLE III.

Determination of Glucose Added to Artificial Dogfish Blood.

Blood sample.	Added glucose.	Reduction values (mg. per 100 cc. blood).			
		Folin.	Error.	Folin-Wu.	Error.
	mg. per 100 cc. blood				
A		7		12	
	16	22	−1	28	0
	25	31	−1	38	+1
	41	48	0	52	−1
	48	53	−2	59	−1
	83	90	0	95	0
B		23		30	
	25	49	+1	54	−1
	50	72	−1	79	−1

From the examples shown in Table II it is evident that excess of oxalate has a marked effect upon the reduction values greater than 100 mg. per 100 cc. of blood. For values of less than 100 mg. the effect is not so striking and suggests that where the amount of oxalate does not greatly exceed 2 mg. per cc. of blood, the reduction value may not be lowered beyond the limit of error. To account for most of the differences between the Folin and Benedict values in Table I, the assumption would have to be made that the blood in these cases contained more than 5 mg. of oxalate per cc.; such was never the case.

There still remained the possibility that by the method used for the preparation of the blood filtrate in the case of dogfish blood

TABLE IV.

Reducing Substances in the Blood of Various Fishes, Expressed as Glucose.

Species.	Common name.	Sex.	Time in tank.	Reducing substances.		Remarks.
				Folin.	Benedict.	
			days	*mg. per 100 cc. blood*	*mg. per 100 cc. blood*	
(*a*) Cyclostomata. *Entosphenus tridentatus.*	Lamprey.	♀	3	64	68	Mixed blood (8 fish).
(*b*) Elasmobranchii. *Squalus sucklii.*	Dogfish.	♀	2	29	34	
		♀	2	30	34	
		♂	2	15	19	
		♀	3	23		
		♀	3	38	48	
		♂	4	55	64	Mixed (4 fish).
		♀	5	54	60	Mixed (2 fish).
		♀	7	52	63	
		♀	7	56	62	
		♀	8	75	86	
(*c*) Holocephali. *Hydrolagus colliæi.*	Ratfish.	♀	1	27	33	
(*d*) Teleostei. *Oncorhynchus kisutch.*	Coho salmon.	♂	2	90	91	
Leptocottus armatus.	Bullhead.	♂	2	29	35	
Sebastodes maliger.	Rock cod.	♂	2	26		Mixed (2 fish).
		♂	2	34		Mixed (3 fish).
Ophiodon elongatus.	Ling cod.	♂	3	34	44	
		♂	5	19		
		♀	5	14	21	
		♂	5	14	23	
		♂	7	10	16	
Pleuronichthys cænosus.	Flounder.	♀	5	18	19	
Platichthys stellatus.	Starry flounder.	♂	2	82		
Porichthys notatus.	Singing-fish.	♀	12	29	31	Mixed (2 fish).

(1 volume of blood + 5 volumes of water + 0.5 volume of each of the precipitating agents) glucose was not estimated quantitatively. In Table III are shown the results of added glucose in such a filtrate prepared from mammalian blood. In order that this blood might approximate as closely as possible to that of the dogfish, mammalian blood obtained after insulin injection was allowed to stand until glycolysis had further reduced the glucose content, and was then diluted with an equal volume of sugar-free Ringer's solution containing added urea, creatine, and creatinine so that the final (diluted) blood contained 2 gm. of urea, 25 to 30 mg. of creatine, and 6 to 8 mg. of creatinine per 100 cc. (*cf.* Denis (8)).

These results show that the method of preparation of the blood filtrate does not appear to be in itself a source of error, nor does the presence of large amounts of nitrogenous solutes influence the estimation of glucose by the Folin method.

In Table IV are shown the amounts of reducing material found in the blood of fishes of different species. Since no explanation has been found to account for the differences in reducing values as determined by the Benedict and the revised Folin procedures, in the majority of cases the results obtained by both methods have been recorded.

The figures in Table IV for dogfish blood appear to be of interest in view of the conflicting statements in the literature. The very low or negative results of Diamare (9), Bierry and Fandard (10), and Lang and Macleod (11) are difficult to reconcile with the average values of 65 and 90 to 110 mg. per 100 cc., recorded by Scott (2) and by Denis (8) respectively. From an examination of the figures in Table IV it would appear that the sugar values obtained bear a definite relationship to the length of time the fish were kept in the tank before analysis, there being apparently an increase to the 4th day. Of the fourteen dogfish whose sugar results are reported, nine were kept from 4 to 8 days and the figures for these are in moderate agreement, giving the (weighted) average value of 57 mg. per 100 cc. Hence a figure of 55 to 60 mg. per 100 cc. is considered to be the probable normal fasting figure. The ling cod was the only other fish kept for varying lengths of time, and in this case the blood sugar appeared to vary in the opposite direction.

A possible explanation of the low values found in the dogfish

may lie in the fact that these fish were all caught by set line, and were probably in an exhausted condition when obtained, although in no single instance could they be considered moribund. Some support is given to this view by the fact that Scott could find no sugar in the blood of certain dogfish which were in a subnormal condition, while, although Lang and Macleod could only find traces of reducing substances in the blood of dogfish freshly caught by line, they found 34 mg. per 100 cc. in one fish which had been caught by net several days previously. That the dogfish while fasting is apparently able slowly to raise its blood sugar to a constant level, is of some interest, since Kilborn and Macleod (12) have shown that there is relatively little glycogen in the liver, and indeed the gradual nature of the rise renders glycogenolysis an unlikely explanation. Tissue protein may be the source of this sugar, but there is an interesting possibility that the liver oil can be utilized for this purpose, especially since recent work in Professor Macleod's laboratory emphasizes the possibility of formation of glucose from fatty acids (13). It is proposed to test this on some future occasion.

Effect of Yeast Fermentation.

The apparent fact that the sugar content of dogfish blood may become depressed far below its normal fasting level without any noticeably deleterious effects to the fish, suggested the possibility that the "sugar" might not be glucose, and that the effect upon it of yeast fermentation was worth investigating. A series of estimations was accordingly carried out, the technique of Folin and Svedberg (7) being followed, with use of the Folin, Folin-Wu, and, in some cases, Benedict reagents. Blanks for the yeast used (Fleischmann's) were determined with all reagents and the values obtained corrected for these blanks. Table V shows the effect of yeast on the sugar of dogfish blood compared with the effect on the blood of certain other fishes.

Table V shows that the sugar in dogfish blood, as determined by the Folin method, is almost completely fermentable, the reduction values after treatment with yeast when corrected for the blank being insignificant. Such, however, is not the case with the teleosts. The figures are of a sufficiently higher degree than the corresponding ones for elasmobranchs to demonstrate the presence

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in the blood of an appreciable amount of non-fermentable reducing substance.

The corrections deducted for the blank were the mean values of a large number of determinations. These mean values, referred

TABLE V.

Reduction Values of Fish Bloods before and after Treatment with Yeast.

Fish.	Reduction values (mg. per 100 cc. blood).								
	Folin.			Folin-Wu.			Benedict.		
	Before yeast.	After yeast.	Fermented material.	Before yeast.	After yeast.	Fermented material.	Before yeast.	After yeast.	Fermented material.
Dogfish (*Squalus sucklii*).	15	4	11	27	12	15	19	7	12
	23	3	20	27	8	19			
	29	4	25	45	8	37			
	30	4	26	44	7	37			
	38	2	36	52	15	37	48	13	35
	52	2	50						
	54	5	49	74	12	62	60	13	47
	56	4	52	71	13	58	62	12	50
	64	6	58	86	15	71	74	10	64
Ratfish (*Hydrolagus colliæi*).	27	3	24						
Ling cod (*Ophiodon elongatus*).	14	7	7	28	17	11			
	19	10	9	30	23	7			
	34	14	20	50	17	33			
Rock cod (*Sebastodes maliger*).	26	12	14						
	34	11	23	48	23	25			
Flounder (*Platichthys stellatus*).	82	10	72	99	21	78			

to 100 cc. of blood, were 6 mg. for teleosts (blood diluted 10 times) and 4 mg. for elasmobranchs (blood diluted 7 times). The extremes were 5 and 9 mg. and 3 and 6 mg. respectively. Hence the difference between the mean value actually deducted and the extreme values observed, cannot account for this difference between elasmobranchs and teleosts.

In view of the small number of teleosts used in this experiment, it would be unwise to stress too greatly the significance of these results, but the moderate agreement between these values contrasted with the wide variation in the total reduction values, suggests that in the blood of at least some species of teleostei there are normally present about 10 to 12 mg. per 100 cc. of non-fermentable reducing substances, as determined by the revised Folin method. This compares with the 5 to 6 mg. per 100 cc., which Folin and Svedberg, using this method, found to represent the average amount of non-fermentable reducing substances in human blood. The nature of these substances has not yet been determined.

When determined by the Folin-Wu procedure, the difference between these "rest reduction" values of elasmobranch and teleost blood does not appear so striking, although here again the latter values are of a somewhat higher order. Folin and Svedberg point out that if yeast only removes from blood substances of equal or greater reducing power than glucose, then the amount of fermentable "sugar" theoretically should be the same whichever method is used. Table V shows that in this investigation the revised Folin and Benedict methods yield figures for the amount of fermentable material which show moderate agreement; those with the Folin-Wu method are definitely higher.[1]

[1] A paper by S. R. Benedict has recently appeared in this *Journal* (1928, lxxvi, 457) in which certain results are in substantial agreement with some of those recorded in this communication. By the use of a new reagent he finds that his previous copper reagent does not yield the lowest "sugar" values, thus agreeing with the present discrepancy noted in Table I between the Benedict and revised Folin methods. Using this new reagent he also obtains values for "total reduction" which are usually lower than the amount of fermented material as estimated by the Folin-Wu method, while the figures for fermented material by his new method are very much lower than the corresponding Folin-Wu figures. Similar divergences are shown in Table V. To explain such divergences, the assumption could be made that fish blood contains large amounts of fermentable "sugar" other than glucose, but this does not seem at all probable. Benedict suggests a much more reasonable explanation, "that blood filtrates may contain one or more substances capable of affecting the dissociation of the copper complex employed, so that the copper becomes more readily reduced by one or more of the reducing materials present in blood."

Effect of Asphyxiation.

The effect of asphyxia upon the blood sugar of fishes has been the subject of several investigations, initiated by the statement of Fandard and Ranc (14) that fish were more susceptible than mammals to hyperglycemia under these conditions. Scott (2)

TABLE VI.

Blood Sugar of Dogfish during Progressive Asphyxiation.

Experiment No.	Sex.	Blood sample.	Time in running water.	Time in stagnant water.	Blood sugar (mg. per 100 cc. blood).	
					Folin method.	Folin-Wu method.
			hrs.	*hrs.*		
1	♀	1	1		24	36
		2		1	20	31
		3		2	12	27
2	♀	1	1		20	31
		2	2		22	32
		3		0.5	16	22
		4		1	14	20
3	♂	1	1		24	34
		2	2		20	27
		3		0.5	18	25
		4		1.5	14	20
		5		3	11	15
4	♀	1	2		26	39
		2		1	19	31
5	♂	1	0		11	23
		2	2		10	22
		3		2	7	18
		4		3	7	10

has recorded a rapid rise in the blood sugar of the dogfish, *Mustelis canis*, during the first 4 minutes followed by a decline to normal within 15; McCormick and Macleod (15) obtained hyperglycemia in the sculpin, *Myoxocephalus*, only after 30 to 45 minutes exposure to asphyxial conditions; confirmation of this delayed rise in blood sugar has been afforded by the work of Simpson (16) and of

Menten (1). As a result of the varying sugar content of dogfish blood recorded in Table IV, it was decided to carry out a few experiments upon the effect of asphyxia.

The experiments were carried out in a rectangular zinc tank through which passed a constant flow of fresh sea water, so regulated that the tank always contained about 60 liters of water. At the bottom of the tank was a wooden trough in which the fish was lightly secured with its ventral surface upwards. When a blood sample was required, one end of the trough was raised until the ventral surface of the fish showed above the level of the water, and the blood obtained by syringe from the heart, which was easily reached by inserting the needle under the posterior edge of the shoulder girdle as described by Menten. The blood was treated by the Folin-Wu technique, the quantities being modified as described previously, and the sugar estimated by both the Folin and Folin-Wu procedures. In the majority of cases the fish was kept in running water for 2 hours, during which time two samples were taken; asphyxial conditions were then induced by securing the inlet and outlet and allowing the fish to use up the dissolved oxygen by respiration. The results are shown in Table VI.

Of the five dogfish whose results are recorded in Table VI, that in Experiment 5 had only been in the sea water tank for 24 hours after capture, that in Experiment 2 for 2 days, and the others, 3 days. Since it has been shown that at least 4 days must elapse after capture before the blood sugar returns to normal, these fish must be regarded as subnormal as far as sugar content is concerned, but it was thought that this would render a hyperglycemic effect all the more noticeable.

In every case the fish died within 30 minutes of taking the last sample, but so far from there being any evidence of hyperglycemia there was a progressive fall in blood sugar, whether determined by the revised Folin or the Folin-Wu procedures. This may be in part accounted for by dilution following withdrawal of a not negligible percentage of the animal's blood, although Menten using the same type of procedure obtained increasing values with consecutive blood samples from the same animal. Further, Hall, Gray, and Lepkovsky (17), analyzing the blood of a large number of fish (menhaden) after individuals had been subjected to varying periods of asphyxiation, found an increase

in most of the blood constituents which was roughly proportional to the length of time during which they were subjected to asphyxial conditions. This they explain by suggesting that with increasing asphyxia the blood becomes more concentrated, water passing from the blood to the tissues. These authors found, however, that the blood sugar did not show this gradual increase, but fluctuated, and they conclude that other factors have a greater effect upon the sugar content than has asphyxia. The conflicting results which different investigators have thus far obtained suggest the desirability of further work in which the conditions are more stringently controlled and the asphyxia is produced under widely varying experimental conditions, and that in the meantime the effect of asphyxia upon the blood sugar of fishes cannot be definitely stated.

SUMMARY.

The blood sugar content under fasting conditions has been determined for fish of different species.

The Folin-Wu procedure for the estimation of the blood sugar has been compared upon a number of fish bloods with the Benedict and Folin modifications. The last named gave distinctly lower values than the Benedict procedure and both much lower values than the original Folin-Wu method. Of the three methods, the revised Folin procedure, when all necessary precautions are observed, and in particular, when the amount of oxalate added to the blood as anticoagulant is carefully regulated, apparently gives results which most closely approximate to the true glucose content.

Blood samples taken from the dogfish, *Squalus sucklii*, shortly after capture, show a low sugar content, but if the fish is kept for a period of not less than 4 days, even under fasting conditions the sugar content rises to an approximately constant level. A value of 55 to 60 mg. per 100 cc. of blood is regarded as the probable normal fasting figure.

Blood filtrates subjected to fermentation with yeast, according to the procedure of Folin and Svedberg, demonstrate that in dogfish blood the sugar is almost completely fermentable, while in the blood of three species of teleosts there appear to be normally present non-fermentable reducing substances to the extent of

about 10 to 12 mg. per 100 cc., as determined by the revised Folin procedure.

Dogfish subjected to conditions of gradually increasing asphyxiation have shown no evidence of hyperglycemia under the experimental conditions employed.

I desire to express my indebtedness to the Biological Board of Canada and the Director of the Pacific Station (Dr. W. A. Clemens) for the facilities placed at my disposal during the course of this investigation. My thanks are also due to Professor A. T. Cameron of this University for his helpful criticism.

BIBLIOGRAPHY.

1. Menten, M. L., *J. Biol. Chem.*, 1927, lxxii, 249.
2. Scott, E. L., *Am. J. Physiol.*, 1921, lv, 349.
3. Folin, O., *J. Biol. Chem.*, 1926, lxvii, 357.
4. Benedict, S. R., *J. Biol. Chem.*, 1925, lxiv, 207.
5. Benedict, S. R., *J. Biol. Chem.*, 1926, lxviii, 759.
6. Harned, B. K., *J. Biol. Chem.*, 1925, lxv, 555.
7. Folin, O., and Svedberg, A., *J. Biol. Chem.*, 1926, lxx, 405.
8. Denis, W., *J. Biol. Chem.*, 1922, liv, 693.
9. Diamare, V., *Arch. ital. biol.*, 1911, lv, 97.
10. Bierry, H., and Fandard, L., *Compt. rend. Acad.*, 1912, cliv, 1717, cited by Lang and Macleod (11).
11. Lang, R. S., and Macleod, J. J. R., *Quart. J. Exp. Physiol.*, 1920, xii, 331.
12. Kilborn, L. G., and Macleod, J. J. R., *Quart. J. Exp. Physiol.*, 1920, xii, 317.
13. Macleod, J. J. R., Carbohydrate metabolism and insulin, London and New York, 1926, 93, 126.
14. Fandard, L., and Ranc, A., *Compt. rend. Soc. biol.*, 1914, lxxvi, 68.
15. McCormick, N. A., and Macleod, J. J. R., *Proc. Roy. Soc. London, Series B*, 1925, xcviii, 1.
16. Simpson, W. W., *Am. J. Physiol.*, 1926, lxxvii, 409.
17. Hall, F. G., Gray, I. E., and Lepkovsky, S., *J. Biol. Chem.*, 1926, lxvii, 549.

LACTONE FORMATION OF CELLOBIONIC AND OF GLUCOARABONIC ACIDS AND ITS BEARING ON THE STRUCTURE OF CELLOBIOSE.

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A systematic study of the structure of disaccharides was undertaken under the incentive of the St. Andrews School, which, under the leadership of Purdie has developed the method of methylation of polyhydric alcohols and of sugars. Over 25 years have passed since the method was introduced, and even to-day, there is no complete harmony between the views of individual workers on the structure of the common disaccharides.

In 1926 Géza Zemplén emphasized the necessity of introducing less drastic methods than those of the St. Andrews School for the study of the structure of disaccharides. Zemplén then suggested a way of progressive degradation of aldobioses until a substance was obtained which no longer was capable of forming an osazone. It was then assumed by him that in the latter substance the hydrogen of the hydroxyl of carbon atom (2) was substituted by the second sugar radicle. The method undoubtedly is very important and ingenious. It has, however, one weak point; namely, that the final conclusion rests on a negative result, which sometimes may be accidental.

A still simpler method was suggested by Levene which is based on the observation of Levene and Simms[1] that each non-substituted sugar acid in solution passes into $< 1, 4 >$ and $<1, 5>$ lactones, and that the velocity of formation of each lactone is a function of the ring structure. With this information the structure

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[1] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxv, 31.

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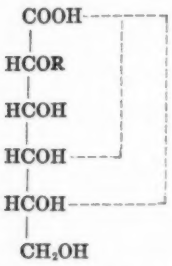
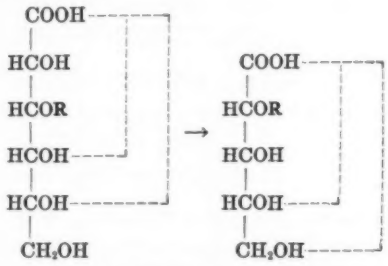
of a disaccharide can be determined from observations on the mutarotation of the bionic acid obtained on oxidation of the disaccharide and of the bionic acid obtained on removing carbon atom (1) from the original disaccharide. The argument may be best illustrated by the following figures.



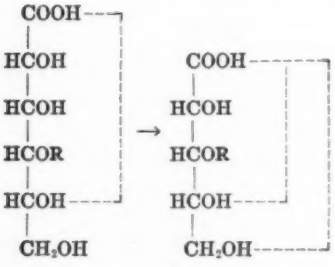
The hexonic acid gives two lactones. It cannot be degraded to a pentose.

I.



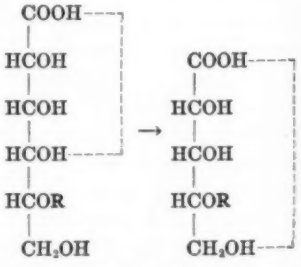
Both the hexonic acid and the corresponding pentonic acid give two lactones.

II.



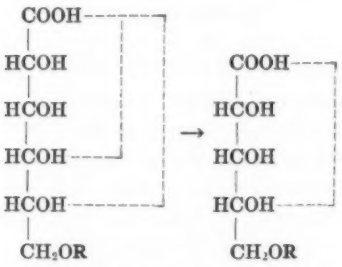
The hexonic acid gives one lactone, < 1, 5 >, and the corresponding pentonic acid two lactones.

III.



The hexonic acid gives one lactone, < 1, 4 >, and the corresponding pentonic acid one lactone, < 1, 5 >.

IV.



The hexonic acid gives two lactones and the corresponding pentonic acid only one, < 1, 4 >.

V.

The work on this plan was begun by Levene and Sobotka[2] in 1926 and was continued by Levene and Wintersteiner[3] in 1927. The present investigation on the structure of cellobiose was begun in 1927 prior to the publication of the article of Haworth, Long, and Plant.[4] From Tables I to IV and from Figs. 1 and 2 it is seen that cellobionic and the corresponding glucose pentonic acids behave like the substance of formula (III) and that therefore in cellobiose the reducing sugar is substituted in position (4). This conclusion is in harmony with those of Zemplén,[5] Irvine,[6] and Haworth, Long, and Plant.[4]

In the course of the present work an improvement was introduced in the purification of α-cellobiose octaacetate, a procedure was given for obtaining the oxime of cellobiose in crystalline form, an improvement was introduced in the method of the conversion of the oxime into the nitrile octaacetate, a procedure was given for the preparation of the calcium salt of glucoarabonic acid in crystalline form, and also a procedure was developed for the preparation of basic lead acetate suitable for the precipitation of bionic acids and for some monocarboxylic sugar acids.

[2] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926-27, lxxi, 471.

[3] Levene, P. A., and Wintersteiner, O., *J. Biol. Chem.*, 1927, lxxv, 315.

[4] Haworth, W. N., Long, C. W., and Plant, J. H. G., *J. Chem. Soc.*, 1927, cxxxi, 2809.

[5] Zemplén, G., *Ber. chem. Ges.*, 1926, lix, 1254.

[6] Irvine, J. C., *Chem. Rev.*, 1927, iv, 203.

EXPERIMENTAL.

Preparation of Calcium Cellobionate.

Crude α-cellobiose octaacetate, prepared from cellulose, was recrystallized several times from a mixture of 1 part of glacial acetic acid and 5 parts of 96 per cent alcohol. The addition of the acetic acid requires the use of less alcohol and gives about the same yield as when alcohol alone is used. The first recrystallization was easily effected by dissolving the material in 12 parts of the hot solvent mixture instead of the 30 parts required when alcohol alone is used. Further recrystallizations require the higher amount of solvent. The purified material showed a melting point of 222.5° and the following specific rotation in chloroform.

$$[\alpha]_D^{22} = \frac{+ 3.23° \times 100}{2 \times 4.02} = + 40.2°.$$

Hudson and Johnson[7] give the m.p. 229.5° and $[\alpha]_D^{22} = + 41°$ in chloroform for highly purified α-cellobiose octaacetate.

The octaacetate was deacetylated with sodium methylate according to the method of Zemplén[5] and the sugar recrystallized from alcohol and water. This material, dried in a vacuum oven, showed a specific rotation 13 minutes after solution of +22° and a final equilibrium value in water as follows:

$$[\alpha]_D^{22} = \frac{+ 1.55° \times 100}{1 \times 4.43} = + 35.0°.$$

Hudson and Yanovsky[8] give the equilibrium value +35° for pure cellobiose and the value +26° 10 minutes after solution.

Cellobionic acid and a number of its salts were first prepared in amorphous condition by Maquenne and Goodwin.[9] We employed the general method of Willstätter and Schudel as modified by Goebel[10] in oxidizing cellobiose to the bionic acid. 18 gm. (1

[7] Hudson, C. S., and Johnson, S. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 1276.

[8] Hudson, C. S., and Yanovsky, E., *J. Am. Chem. Soc.*, 1917, xxxix, 1035.

[9] Maquenne, L., and Goodwin, W., *Bull. Soc. chim.*, 1904, xxxi, 854.

[10] Goebel, W. F., *J. Biol. Chem.*, 1927, lxxii, 801.

mol) of cellobiose were dissolved in 700 cc. of a solution containing 27 gm. (2 mols) of iodine and 50 gm. of barium iodide. To this was added over an interval of 5 to 10 minutes, with mechanical stirring, a solution of 50 gm. (3 mols) of barium hydrate in 800 cc. of water. The solution was allowed to stand 15 minutes, after which a solution of 18 cc. of concentrated sulfuric acid in 130 cc. of water was added under strong mechanical stirring. This was followed by the immediate addition of 220 gm. of washed basic lead carbonate. Under continual stirring the solution soon became neutral to Congo red. The precipitate was allowed to settle, filtered on a prepared bed of kieselguhr and charcoal, and the filtrate concentrated under reduced pressure to about 400 cc. The solution was filtered from the separated lead iodide; most of the lead was removed with sulfuric acid and the iodide ion with silver sulfate. After filtration, the remaining lead and silver were removed with hydrogen sulfide, the hydrogen sulfide by air, and the solution again filtered. In the filtrate the excess sulfate ion was removed quantitatively with barium hydroxide and the solution filtered. The filtrate was shaken with an excess of well washed, precipitated calcium carbonate for 15 minutes. Short boiling completed the formation of the calcium salt. The solution was cooled, filtered, and concentrated under reduced pressure to a thin syrup. This was poured into 12 to 15 volumes of anhydrous methyl alcohol. The salt precipitated as a dense, amorphous solid. This was taken up in about 3 times its weight of water and precipitated as a syrup by the addition of 1½ volumes of 96 per cent alcohol. The supernatant liquid was decanted, the residue dissolved in a small amount of water, and poured into 10 to 15 volumes of absolute methyl alcohol. This procedure was repeated. Due to the high solubility of this salt, only 2.5 gm. of purified material were obtained. This, as well as the cadmium and barium salts, resisted repeated attempts at crystallization. The salt analyzed as follows:

6.499 mg. substance: 11.65 mg. $CaSO_4$.

0.1456 gm. dried substance: 0.2028 gm. CO_2 and 0.0720 gm. H_2O. Moisture, 7.30 per cent.

$Ca(C_{12}H_{21}O_{12})_2$.	Calculated.	C 38.18, H 5.61, Ca 5.31.
	Found.	" 37.98, " 5.53, " 5.30.

The salt was kept in a weighed container over calcium chloride and subsequent weighings were corrected for a slow loss of moisture.

TABLE I.

Changes in Rotation of 0.090 N Cellobionic Acid Solution.

Experiment 1. l = 2 dm., t = 22°, λ = 5892 Å.			Experiment 2. l = 4 dm., t = 23°, λ = 5892 Å.		
Time.	α	$[\alpha]_D$	Time.	α	$[\alpha]_D$
min.	*degrees*	*degrees*	*min.*	*degrees*	*degrees*
			1	−0.41	−3.2
2	−0.21	−3.3	2	−0.35	−2.7
6	−0.15	−2.3	6	−0.33	−2.5
8	−0.14	−2.2	8	−0.30	−2.3
15	−0.12	−1.9	23	−0.15	−1.2
30	−0.09	−1.4	32	−0.06	−0.5
45	−0.04	−0.6	45	−0.02	−0.2
hrs.			*hrs.*		
1	+0.02	+0.3	1	+0.03	+0.2
2	+0.05	+0.8	2	+0.09	+0.7
4	+0.05	+0.8	4	+0.13	+1.0
6	+0.05	+0.8	6	+0.13	+1.0
20	+0.06	+0.9	24	+0.14	+1.1

TABLE II.

Titration of 1.00 Cc. of 0.090 N Cellobionic Acid Solution with 0.1 N Sodium Hydroxide.

Experiment 1. t = 22°		Experiment 2. t = 23°	
Time.	0.1 N NaOH.	Time.	0.1 N NaOH.
hrs.	*cc.*	*hrs.*	*cc.*
1	0.90	1	0.90
2	0.89		
5	0.89	5	0.90
8	0.90		
20	0.89	23	0.91

Mutarotation and Titration Experiments.

A weight of 0.9020 gm. of calcium cellobionate containing 5.44 per cent of water, thus corresponding to 0.8529 gm. of dry substance, was dissolved in about 15 cc. of water. The calculated

amount, 4.52 ± 0.01 cc. of 0.5 N hydrochloric acid was added and the solution quickly made up to 25.00 cc. The solution was thus 0.0905 N. The change in rotation was observed and samples of 1.00 cc., obtained with an accurate micro pipette, were titrated at suitable intervals with 0.1 N sodium hydroxide (phenolphthalein) delivered from a micro burette. Care was exercised that the water used in the experiments did not contain sufficient carbon dioxide to influence the end-point. The data are recorded in Tables I and II and the polarimetric change obtained is plotted in Fig. 1.

The interpolated initial value for the specific rotation of the free acid is −3.6°. At the time of the last reading the solution gave no reduction with Fehling's solution, showing that no apparent hydrolysis had taken place.

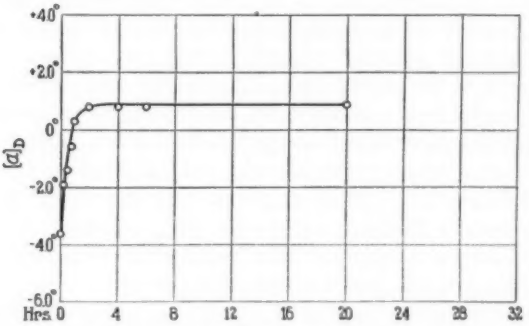


FIG. 1. <1,5> lactone formation of cellobionic acid. Experiment 1.

Preparation of Calcium Glucoarabonate from Cellobiose.

I. Preparation of Crystalline Cellobiose Oxime.—For the preparation of glucoarabinose the procedure of Zemplén[5] was followed with some modification. We were able to prepare cellobiose oxime in crystalline condition. The procedure of Zemplén was followed except that we used pure (99 per cent by Raschig's reduction procedure) hydroxylamine hydrochloride and removed the excess hydroxylamine, after oxime formation, by distillation under reduced pressure, in the presence of solid calcium carbonate to neutralize any acidity. The quantities used by Zemplén in the decomposition of the hydrochloride with sodium methylate leave the former

in slight excess. The crude residue was dried by distillation with absolute alcohol. On nucleation the material crystallized. This material was then stirred with warm 96 per cent alcohol, cooled in an ice-salt mixture, filtered, washed with absolute alcohol, and dried. For the purposes of the next step this material is sufficiently pure. A product more nearly pure was obtained as follows: The crude evaporated product from 43 gm. of cellobiose was dissolved in 3 to 4 times its weight of water, decolorized at room temperature, and filtered. To the filtrate acetone was added with stirring to opalescence. The solution was then nucleated and allowed to stand, first at room temperature and then for some time in the refrigerator. It was filtered, washed with 80 per cent acetone, and then with pure acetone. A weight of 24.2 gm., m.p. 122–123°, was so obtained. The mother liquor was concentrated to 50 cc. and the procedure repeated, 8.2 gm. of product melting at 119–120° being obtained. The filtrate was concentrated to a thin syrup and treated with hot 96 per cent alcohol to slight turbidity, nucleated, and kept overnight in the refrigerator. A further quantity of 1.5 gm. of product melting at 119–120° was obtained, making a total yield of 76 per cent.

Cellobiose oxime crystallizes in plates, is very soluble in water, slightly soluble in cold methyl and ethyl alcohols, moderately soluble in warm methyl and ethyl alcohols, and insoluble in acetone, ether, benzene, ethyl acetate, and petroleum ether (40–60°). On one recrystallization from 80 per cent ethyl alcohol the material melted at 123–125°. A second recrystallization from the same solvent gave material melting at 123–125° and having $[\alpha]_D^{22} = -26.1°$ (1.0313 gm. of substance, 25 cc. of aqueous solution, $\alpha = -2.15°$, 2 dm. tube) in aqueous solution, changing at a very slow rate in the dextro direction. A third recrystallization gave material melting at 123–125° and showing the initial $[\alpha]_D^{24} = -26.1°$ (1.1131 gm. of substance, 25 cc. of aqueous solution, $\alpha = -2.32°$, 2 dm. tube). This purified material analyzed as follows:

5.310 mg. substance: 0.185 cc. N_2 (760 mm., 24°).

$C_{12}H_{23}O_{11}N$. Calculated. N 3.92.
Found. " 4.00.

II. Preparation of Cellobionic Acid Nitrile Octaacetate.—The procedure of Zemplén calls for the acetylation of the syrupy oxime

reaction mixture and in our experience always produced a violent reaction with loss of material and resin formation. With the crystalline oxime this reaction may be easily controlled by the following procedure. A mixture of 140 cc. of acetic anhydride and 21 gm. of fused sodium acetate was heated in an oil bath to 110°. The crystalline oxime (21 gm.) was then added in small portions. Reaction began immediately and the mixture was removed from the oil bath. The addition was continued, the rate being regulated so as to maintain the mixture at 110–115°, and the reaction's own heat being utilized. The addition required about half an hour and was followed by 1 hour of heating at 110°. The preparation was finished according to the directions of Zemplén, the recrystallized product melting at 132° in agreement with Zemplén. It is suggested for similar procedures with other sugars that, where the oxime does not crystallize, it be added gradually to the acetylating mixture in the form of an amorphous powder.

III. Preparation of Calcium Glucoarabonate from Glucoarabinose Heptaacetate.—Glucoarabinose heptaacetate was prepared from cellobionic acid nitrile octaacetate according to the directions of Zemplén. The recrystallized material melted at 194° and showed the following rotation in chloroform.

$$[\alpha]_D^{22} = \frac{-1.20° \times 100}{2 \times 4.42} = -14°.$$

Zemplén records the melting point of 196° and $[\alpha]_D^{15} = -16.95°$ in chloroform solution. 12.5 gm. of the acetylated sugar were deacetylated according to the directions of Zemplén and the resulting solution of sugar and sodium acetate concentrated to 40 cc. to remove methyl alcohol. This solution was oxidized to the aldobionic acid by exactly the same procedure as was previously described for the preparation of cellobionic acid. The amounts of oxidizing reagents used were 19 gm. of iodine, 37 gm. of barium iodide in 500 cc. of water, and 46 gm. of purified barium hydrate in 725 cc. of water, 12 minutes being taken for the addition of the alkali. After removal of the silver and lead ions, the solution containing the oxidation product together with a considerable amount of sodium acetate was neutralized with barium hydroxide solution until the solution was just acid to litmus. The

in slight excess. The crude residue was dried by distillation with absolute alcohol. On nucleation the material crystallized. This material was then stirred with warm 96 per cent alcohol, cooled in an ice-salt mixture, filtered, washed with absolute alcohol, and dried. For the purposes of the next step this material is sufficiently pure. A product more nearly pure was obtained as follows: The crude evaporated product from 43 gm. of cellobiose was dissolved in 3 to 4 times its weight of water, decolorized at room temperature, and filtered. To the filtrate acetone was added with stirring to opalescence. The solution was then nucleated and allowed to stand, first at room temperature and then for some time in the refrigerator. It was filtered, washed with 80 per cent acetone, and then with pure acetone. A weight of 24.2 gm., m.p. 122–123°, was so obtained. The mother liquor was concentrated to 50 cc. and the procedure repeated, 8.2 gm. of product melting at 119–120° being obtained. The filtrate was concentrated to a thin syrup and treated with hot 96 per cent alcohol to slight turbidity, nucleated, and kept overnight in the refrigerator. A further quantity of 1.5 gm. of product melting at 119–120° was obtained, making a total yield of 76 per cent.

Cellobiose oxime crystallizes in plates, is very soluble in water, slightly soluble in cold methyl and ethyl alcohols, moderately soluble in warm methyl and ethyl alcohols, and insoluble in acetone, ether, benzene, ethyl acetate, and petroleum ether (40–60°). On one recrystallization from 80 per cent ethyl alcohol the material melted at 123–125°. A second recrystallization from the same solvent gave material melting at 123–125° and having $[\alpha]_D^{25} = -26.1°$ (1.0313 gm. of substance, 25 cc. of aqueous solution, $\alpha = -2.15°$, 2 dm. tube) in aqueous solution, changing at a very slow rate in the dextro direction. A third recrystallization gave material melting at 123–125° and showing the initial $[\alpha]_D^{24} = -26.1°$ (1.1131 gm. of substance, 25 cc. of aqueous solution, $\alpha = -2.32°$, 2 dm. tube). This purified material analyzed as follows:

5.310 mg. substance: 0.185 cc. N_2 (760 mm., 24°).

$C_{12}H_{22}O_{11}N$.	Calculated.	N 3.92.
	Found.	" 4.00.

II. Preparation of Cellobionic Acid Nitrile Octaacetate.—The procedure of Zemplén calls for the acetylation of the syrupy oxime

reaction mixture and in our experience always produced a violent reaction with loss of material and resin formation. With the crystalline oxime this reaction may be easily controlled by the following procedure. A mixture of 140 cc. of acetic anhydride and 21 gm. of fused sodium acetate was heated in an oil bath to 110°. The crystalline oxime (21 gm.) was then added in small portions. Reaction began immediately and the mixture was removed from the oil bath. The addition was continued, the rate being regulated so as to maintain the mixture at 110–115°, and the reaction's own heat being utilized. The addition required about half an hour and was followed by 1 hour of heating at 110°. The preparation was finished according to the directions of Zemplén, the recrystallized product melting at 132° in agreement with Zemplén. It is suggested for similar procedures with other sugars that, where the oxime does not crystallize, it be added gradually to the acetylating mixture in the form of an amorphous powder.

III. Preparation of Calcium Glucoarabonate from Glucoarabinose Heptaacetate.—Glucoarabinose heptaacetate was prepared from cellobionic acid nitrile octaacetate according to the directions of Zemplén. The recrystallized material melted at 194° and showed the following rotation in chloroform.

$$[\alpha]_D^{23} = \frac{-1.20° \times 100}{2 \times 4.42} = -14°.$$

Zemplén records the melting point of 196° and $[\alpha]_D^{16} = -16.95°$ in chloroform solution. 12.5 gm. of the acetylated sugar were deacetylated according to the directions of Zemplén and the resulting solution of sugar and sodium acetate concentrated to 40 cc. to remove methyl alcohol. This solution was oxidized to the aldobionic acid by exactly the same procedure as was previously described for the preparation of cellobionic acid. The amounts of oxidizing reagents used were 19 gm. of iodine, 37 gm. of barium iodide in 500 cc. of water, and 46 gm. of purified barium hydrate in 725 cc. of water, 12 minutes being taken for the addition of the alkali. After removal of the silver and lead ions, the solution containing the oxidation product together with a considerable amount of sodium acetate was neutralized with barium hydroxide solution until the solution was just acid to litmus. The

barium sulfate was removed by filtration and the filtrate concentrated under reduced pressure at 40° to a volume of 40 cc. A saturated solution of basic lead acetate was added to the solution until no further precipitation occurred. The voluminous precipitate so obtained was filtered and washed with a small amount of very cold water. To the filtrate barium hydroxide was added and a further quantity of precipitate obtained, which was combined with the first.

Fischer and Meyer[11] used basic lead acetate in the preparation of maltobionic acid. They stated that the common form of basic lead acetate did not precipitate the acid and described a method of preparing a basic compound suitable for this purpose. We were unable to obtain active material by repeating their procedure. The right form of basic lead acetate may easily be prepared in crystalline condition by adding barium hydroxide to a solution of ordinary basic lead acetate until a precipitate just begins to form. The solution is then concentrated until a separation of the active basic lead acetate in well formed needles takes place. These are removed by filtration.

The combined precipitates of the basic lead salt of the sugar acid were suspended in about 500 cc. of water and hydrogen sulfide passed into the solution. The solid material was filtered, ground in a mortar, and resuspended in the original solution, and again treated with hydrogen sulfide. This process was repeated once. The solution was filtered from the lead sulfide and the hydrogen sulfide removed from the filtrate with a current of air. A trace of barium was removed with sulfuric acid and the most of a small amount of acetic acid present removed by repeated extraction with a considerable volume of chloroform. The filtered solution was shaken with an excess of precipitated and washed calcium carbonate for 15 minutes, boiled 8 minutes, and allowed to stand overnight. After filtration, the solution was concentrated at 40° to a thin syrup and this poured into 10 to 15 volumes of absolute methyl alcohol. The amorphous material was dissolved in water and a mixture of methyl alcohol and acetone added to opalescence. On heating this mixture the material crystallized in radiating clusters of needles. The recrystallization process was repeated,

[11] Fischer, E., and Meyer, J., *Ber. chem. Ges.*, 1889, xxii, 1941.

TABLE III.

Changes in Rotation of 0.112 N Glucoarabonic Acid Solution.

l = 4 dm. λ = 5892 Å

Experiment 1. t = 22°			Experiment 2. t = 23°		
Time.	α	$[\alpha]_D$	Time.	α	$[\alpha]_D$
min.	*degrees*	*degrees*	*min.*	*degrees*	*degrees*
2.5	+2.94	+20.0			
5	2.87	19.5			
7.5	2.84	19.3	6	+2.75	+18.7
10	2.82	19.2	10	2.74	18.6
15	2.78	18.9	15	2.69	18.3
30	2.71	18.4	30	2.62	17.8
45	2.68	18.2	45	2.56	17.4
hrs.			*hrs.*		
1	2.63	17.9	1	2.54	17.3
1.5	2.56	17.4	1.5	2.51	17.1
2	2.51	17.1	2	2.48	16.9
2.5	2.48	16.9			
3	2.50	17.0	3	2.42	16.5
4	2.52	17.1			
5	2.54	17.3	5	2.51	17.1
6	2.57	17.5	7	2.55	17.3
10	2.65	18.0			
23	2.82	19.2	24	2.86	19.4
29	2.91	19.8			

TABLE IV.

Titration of 1.00 Cc. of 0.112 N Glucoarabonic Acid Solution with 0.1 N Sodium Hydroxide.

Experiment 1. t = 22°		Experiment 2. t = 23°	
Time.	0.1 N NaOH.	Time.	0.1 N NaOH.
hrs.	*cc.*	*hrs.*	*cc.*
0.5	1.10		
1	1.10		
2	1.07	1.5	1.10
4	1.05	5.5	1.05
10	1.03		
30	0.89	24	0.86

The permanent titer found by titrating the hot solution was 1.13 cc.

5 gm. of material being obtained. The analytical results of the material dried first at 100° and then at 110° under reduced pressure over sulfuric acid indicated that it still contained 1 molecule of water. This behavior is similar to that of the crystalline calcium galactoarabonate obtained by Levene and Wintersteiner.[3]

0.1078 gm. substance: 0.0088 gm. CaO.
0.1046 " " : 0.1424 " CO_2 and 0.0514 gm. H_2O.
$Ca(C_{11}H_{19}O_{11})_2 \cdot H_2O$. Calculated. C 37.06, H 5.66, Ca 5.63.
Found. " 37.12, " 5.49, " 5.83.

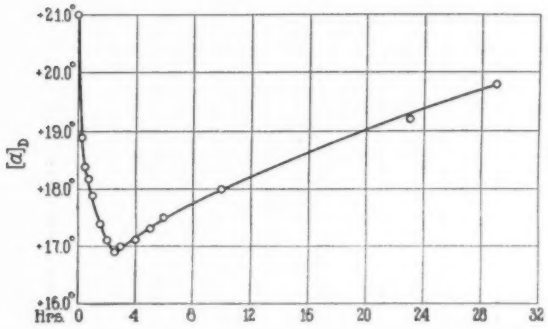


FIG. 2. <1,5> and <1,4> lactone formation of glucoarabonic acid. Experiment 1.

The rotation of the calcium salt was as follows:

$$[\alpha]_D^{25} = \frac{+ 0.27° \times 100}{1 \times 1.88} = + 14.4°.$$

Another preparation gave the following result.

$$[\alpha]_D^{21} = \frac{+ 0.43° \times 100}{1 \times 3.03} = + 14.2°.$$

Mutarotation and Titration Experiments.

A weight of 1.000 gm. of calcium glucoarabonate containing no water other than the molecule of water of crystallization, was dissolved in about 15 cc. of water. The calculated amount, 5.61 ± 0.01 cc., of 0.5 N hydrochloric acid was added and the solution

quickly made up to 25.00 cc. The solution was thus 0.1123 N. The change in rotation was observed and samples of 1.00 cc. titrated at suitable intervals with 0.1 N sodium hydroxide (phenolphthalein) as described with cellobionic acid. The first endpoint obtained was recorded. The data obtained are listed in Tables III and IV and the polarimetric change is plotted in Fig. 2.

The interpolated initial value for the specific rotation of the free acid is +21°. At the time of the last reading a sample of the solution containing 30 to 40 mg. of the sugar acid gave no reduction with Fehling's solution, showing that no apparent hydrolysis had taken place.

STUDIES IN POLYMERIZATION AND CONDENSATION.*

II. PRODUCTS OF INTERREACTION OF POTASSIUM ACETATE AND EPICHLOROHYDRIN.

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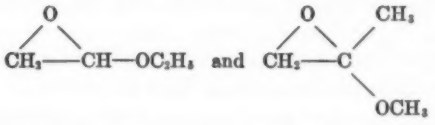
(Received for publication, March 30, 1928.)

Many voices have been raised recently against the indiscriminate use of the term "polymerization." The same term is applied to processes of simple aggregation of molecules and to processes of condensation through forces of primary valence.[1]

The confusion in the use of the term is most striking in its application to ethylene oxidic structures. Thus, even in the third edition of Houben's "Die Methoden der organischen Chemie," we find under the same head the process of condensation of ethylene oxide under the influence of zinc chloride or of potassium hydroxide and the spontaneous association of 2 molecules of half acetals of hydroxyaldehydes or of hydroxyketones, such as



In part the confusion is undoubtedly due to the fact that much of the work in this field dates far in the past when the experimental material was not large and when the details of the structures of the so called polymers remained unknown.

* The first paper of the series is entitled "On Condensation Products of Propylene Oxide and of Glycidol," Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1927, lxxv, 325.

[1] Schroeter, G., *Ber. chem. Ges.*, 1916, xlix, 2697; 1920, liii, 1917. Staudinger, H., *Ber. chem., Ges.*, 1920, liii, 1073. Bergmann, M., *Ann. Chem.*, 1927, edlii, 121.

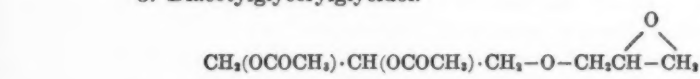
XUM

The reasons for our undertaking the task of clearing up the structure of the group of ethylene oxidic polymers was given in the first article of this series.[2] In that place were described the condensation products of propylene oxide and of glycidol formed under the simplest conditions.

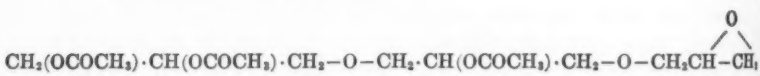
In the present article are described the substances which are formed through the action of dry potassium acetate on epichlorohydrin. The statement was made by Breslauer[3] that under these conditions in addition to glycidol acetate a polymer of glycidol was formed. The directions of this author were followed in our experiments.

By means of fractional distillation the following substances were isolated from the reaction product.

1. Acetylglycidol.
2. Diacetin.
3. Diacetylglycerylglycidol.



4. Triacetyldiglycerylglycidol.



5. Higher condensation products.

Each of these products was identified by its carbon and hydrogen content, by its saponification value, and by its molecular weight. The acetyl derivatives were then deacetylated and the free substances were analyzed and then reacetylated.

Thus, under the above mentioned conditions, acetylglycidol has a tendency to condense into di- and polymolecular substances in the same manner as free glycidol.

EXPERIMENTAL.

Experiment 1.

To 200 gm. of finely pulverized dry potassium acetate were added 150 gm. of freshly distilled epichlorohydrin (b.p. 115–116°).

[2] Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1927, lxxv, 325.
[3] Breslauer, M., *J. prakt. Chem.*, 1879, xx, 188.

This mixture was heated for 27 hours, with shaking at intervals, in an oil bath, under a reflux condenser provided with a calcium chloride tube. The temperature was maintained at 120–135° for the greater part of the time, but toward the end of the experiment it was allowed to rise to 150°. After cooling, the reaction product was extracted with dry ether. The ether was removed by distillation and the residue was fractionated into ten fractions.

I. 112–135°, at ordinary pressure; mostly unchanged epichlorohydrin; about 35 gm.
II. 45– 73°, at 25 mm.; mixture of epichlorohydrin and glycidol acetate; 18 gm.
III. 74– 76°, at 25 mm., glycidol acetate; 35 gm.
IV. 45– 97°, " 0.5 to 1 mm.; 8 gm.
V. 96–100°, " 0.5 mm.; diacetin; 8 gm.
VI. 102–111°, " 0.5 " 2 gm.
VII. 114–125°, " 0.5 " diacetylglycerylglycidol; 6 gm.
VIII. 130–140°, " 0.5 " 2 gm.
IX. 138–155°, " 0.2 " 2 "
X. 153–165°, " 0.2 " 8 "

Fraction V.—This fraction was redistilled and the part boiling at 87–93°, at 0.03 to 0.05 mm., was analyzed. It had the composition of diacetin.

0.0841 gm. substance: 0.1474 gm. CO_2 and 0.0534 gm. H_2O.
$C_7H_{12}O_5$. Calculated. C 47.70, H 6.87.
Found. " 47.79, " 7.12.

Saponification Number.—0.2674 gm. of substance was refluxed with 10 cc. of 0.5 N KOH for 3 hours. 19.1 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 637. Found. 648.

Molecular Weight Determination by Method of Menzies and Wright.[4]—(I) 0.2171 gm. of substance dissolved in 29.7 cc. of benzene (b.p. 80.0°, at 755 mm.) gave an elevation of 27 mm. on the differential thermometer. (II) 0.3493 gm. of substance dissolved

[4] Menzies, A. W. C., and Wright, S. L., Jr., *J. Am. Chem. Soc.*, 1921, xliii, 2314.

in 31.0 cc. of benzene (b.p. 80.5°, at 763 mm.) gave an elevation of 42 mm. on the differential thermometer.

Molecular weight. Calculated. 176. Found. I. 178.
II. 176.

Hydrolysis of Fraction V.—This substance was hydrolyzed in the following manner. 6 gm. of the material were refluxed over a free flame with 30 cc. of 5 per cent sulfuric acid. The acetic acid thus formed was extracted by means of ether in a continuous extraction apparatus. The operation was continued 5 hours. From the remaining aqueous solution the sulfuric acid was removed quantitatively by means of a solution of barium hydroxide. The filtrate was then concentrated under reduced pressure and the residue distilled at 130°, at 0.9 mm. Yield 2 gm. The substance had the composition of glycerol.

0.1028 gm. substance: 0.1480 gm. CO_2 and 0.0813 gm. H_2O.
$C_3H_8O_3$. Calculated. C 39.13, H 8.67.
Found. " 39.26, " 8.84.

Acetylation of the Non-Hydrolyzed Substance.—To 3.2 gm. of the above described reaction product (diacetin) were added 10 cc. of acetic anhydride and the mixture was refluxed for 3½ hours. The excess of the anhydride and acetic acid was removed under reduced pressure (10 to 15 mm.). The fraction boiling from 96–98°, at 1.0 mm., was collected. It weighed 1.8 gm. and analyzed for triacetin.

0.0799 gm. substance: 0.1446 gm. CO_2 and 0.0464 gm. H_2O.
$C_3H_5(OCOCH_3)_3$. Calculated. C 49.54, H 6.42.
Found. " 49.35, " 6.49.

Saponification Number.—0.2156 gm. of substance was refluxed for 3 hours with 10.0 cc. of 0.5 N KOH. 21.1 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 772. Found. 752.

Fraction VII.—This fraction (representing diacetylglyceryl-glycidol) was redistilled and the part boiling at 110–114°, at 0.1 mm., was collected. It analyzed as follows:

4.351 mg. substance: 8.310 mg. CO_2 and 2.785 mg. H_2O.
$C_{10}H_{16}O_6$. Calculated. C 51.73, H 6.89.
Found. " 52.08, " 7.16.

Saponification Number.—0.3139 gm. of substance was refluxed for 3 hours with 15 cc. of 0.5 N KOH and 10 cc. of distilled water. 49.1 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 484. Found. 463.

Molecular Weight Determination by Method of Menzies and Wright.—30.0 cc. of benzene (b.p. 79.8°, at 749 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	mm.	
0.1113	10	237
0.2431	22	236
0.3836	35	234
0.5633	51	235

Molecular weight calculated, 232.

Hydrolysis of the Substance into Diglycerol.—6 gm. of this substance were refluxed for 6 hours with 30 cc. of 5 per cent sulfuric acid and 10 cc. of water. The solution was worked up as described above. After neutralization with barium hydroxide the filtrate was concentrated and the viscous residue distilled at 196–197°, at 0.5 to 0.6 mm. The yield was 3 gm. of diglycerol. The substance analyzed as follows:

4.589 mg. substance: 7.370 mg. CO_2 and 3.565 mg. H_2O.
$C_6H_{14}O_5$. Calculated. C 43.35, H 8.49.
Found. " 43.79, " 8.69.

Crystalline Diacetylglycerylglycidol from Fraction VII.—On standing for several weeks a crystalline deposit appeared in this fraction. The crystals were filtered off and recrystallized three times from benzene to which a little ligroin was added. After drying at 80°, under reduced pressure, for 14 hours the substance melted at 125°. It analyzed as follows:

5.940 mg. substance: 11.290 mg. CO_2 and 3.800 mg. H_2O.
$C_{10}H_{16}O_6$. Calculated. C 51.73, H 6.89.
Found. " 51.83, " 7.15.

Saponification Number.—0.0887 gm. of substance was refluxed for 1½ hours with 40 cc. of 0.1 N KOH. 32.4 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 484. Found. 481.

Molecular Weight Determination by Method of Menzies and Wright.—31.5 cc. of benzene (b.p. 80.0°, at 758 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.1454	13	229
0.2710	24	231

Molecular weight calculated, 232.

Fraction X.—7.0 gm. of the substance boiling at 153–165°at 0.2 mm. pressure were hydrolyzed with 30 cc. of 5 per cent sulfuric acid. 3.0 gm. of diglycerol boiling at 192–193° at 0.2 to 0.3 mm. were obtained. The analysis was as follows:

6.950 mg. substance: 11.400 mg. CO_2 and 5.365 mg. H_2O.
$C_6H_{14}O_4$. Calculated. C 43.35, H 8.49.
Found. " 43.24, " 8.63.

Experiment 2.

In this experiment the time of reaction was extended to 48 hours. To 450 gm. of powdered dry potassium acetate were added 370 gm. of freshly distilled epichlorohydrin. The mixture was heated at 125–130° for 24 hours, at 130–140° for 12 hours, and at 140–155° for 12 hours, and then extracted with ether. The ether was removed by distillation and from the residue the following fractions were obtained.

I. 110–120°, at ordinary pressure; unchanged epichlorohydrin; 70 gm.
II. 70– 80°, " 21 mm.; crude glycidol acetate; 40 gm.
III. 35– 95°, " 1 " 6 gm.
IV. 95–110°, " 0.7 " 6 "
V. 108–130°, " 0.8 " 35 "
VI. 128–155°, " 0.3 " 25 "

At this point the distillation was interrupted. After 2 hours it was resumed. The part which came over at 90°, at 0.3 mm.

pressure, contained platelets which continuously increased in size until some of the crystals reached the length of 1 inch. They were arranged in rosettes in the upper and side parts of the receiver. About 12 gm. of substance came over before the temperature rose to 165°, at 0.3 mm. (Fraction VII). The final fraction boiled at 165–210°, at 0.2 to 0.5 mm. (Fraction VIII). Some decomposition took place at this temperature. The residue weighed about 120 gm. It was dark in color and reacted neutral to litmus paper, was soluble in benzene, acetone, and alcohol and insoluble in ether and water. Inasmuch as the original material was soluble in ether, it is possible that this product was formed during distillation, although the possibility is not excluded that the product was soluble in ether in the presence of the material which came over in the other fractions.

Fraction Boiling from 128–155° (*Fraction VI.*)—This portion was redistilled with the aid of a small column. From it the following fractions were obtained.

IX. 104–114°, at 0.2 mm.; 5.3 gm.
X. 112–114°, " 0.1 " 6.6 " diacetylglycerylglycidol.
XI. 130–140°, " 0.1 " 3.6 "
XII. 140–155°, " 0.1 " 3.6 "

To the residue was added the portion boiling at 0.2 mm. from 153–165° (Fraction X of Experiment 1). It was then distilled below 100°, at 0.1 mm. 3.5 gm. were collected and at the same time 0.4 gm. of crystals separated. On continuing the distillation the following fractions were obtained.

XIII. 155–168°, at 0.05 to 0.03 mm.; 5.8 gm.
XIV. 200–210°, " 0.3 " 0.6 " 3.7 "

Acetylation of Diglycerol.—All fractions boiling between 112–160°, at 0.03 to 0.3 mm., *i.e.* Fractions VII, VIII, IX, and X of Experiment 1 as well as Fractions X, XI and XII of Experiment 2, yielded diglycerol on acid hydrolysis. There was an indication of the presence of a small quantity of glycerol also. The carbon content of the higher boiling fractions (130–210°, at 0.2 mm. or less) varied little (50 to 51 per cent).

To 3 gm. of the diglycerol obtained from one of these experiments were added 15 cc. of redistilled acetic anhydride and the

mixture was refluxed for 3 hours. The excess of the anhydride was removed under diminished pressure with the aid of a small fractional distilling flask. The fraction boiling from 140–153°, at 0.05 to 0.1 mm., was collected. It analyzed for tetra-acetyldiglycerol.

5.534 mg. substance: 10.253 mg. CO_2 and 3.405 mg. H_2O.
$C_{14}H_{22}O_9$. Calculated. C 50.28, H 6.64.
Found. " 50.52, " 6.88.

Saponification Number.—0.2841 gm. of substance was refluxed for 3 hours with 10 cc. of 0.5 N KOH. 16.25 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 671. Found. 666.

Molecular Weight Determination by Method of Menzies and Wright.—30.1 cc. of benzene (b.p. 80.1°, at 758 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.1576	10	338
0.3597	23	336
0.5507	35	338
0.9522	59	346

Molecular weight calculated, 334.

Fraction XIII.—The fraction boiling from 155–168°, at 0.05 to 0.03 mm., was very probably a mixture of a larger amount of triacetyldiglycerol and an acetylated triglycerol derivative. It analyzed as follows:

0.0885 gm. substance: 0.1622 gm. CO_2 and 0.0542 gm. H_2O.
$C_{12}H_{20}O_8$. (Triacetyldiglycerol). Calculated. C 49.30, H 6.55.
Found. " 49.97, " 6.85.

Saponification Number.—0.2566 gm. of substance was refluxed for 2 hours with 10.5 cc. of 0.5 N KOH. 26.5 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated for triacetyldiglycerol. 576.
Found. 568.

Molecular Weight Determination by Method of Menzies and Wright.—30.0 cc. of benzene (b.p. 80.6°, at 769 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.2843	22	283
0.4620	33	307
0.6402	44	316

Molecular weight calculated for triacetyldiglycerol, 292.

Hydrolysis of Fraction XIII.—3.8 gm. of substance were heated gently for 12 hours with 20 cc. of 5 per cent sulfuric acid. The reaction product was freed from acetic and sulfuric acids and concentrated. The anhydrous residue weighed 1.9 gm. On distillation about 0.2 gm. came over at the boiling point of glycerol while the main fraction distilled at 176–180°, at 0.06 mm. The analysis was as follows:

0.1185 gm. substance: 0.1914 gm. CO_2 and 0.0896 gm. H_2O.
$C_6H_{14}O_5$ (diglycerol). Calculated. C 43.35, H 8.49.
Found. " 44.04, " 8.46.

This would indicate that this fraction represents a mixture of di- and triglycerol.

Fraction XIV.—The fraction boiling at 200–210°, at 0.3 to 0.6 mm., weighed 3.7 gm. and analyzed as follows:

0.1068 gm. substance: 0.1996 gm. CO_2 and 0.0674 gm. H_2O.
Found. C 50.96, H 7.06.

Saponification Number.—0.2109 gm. of substance was refluxed for 2 hours with 10 cc. of 0.5 N KOH. 31.3 cc. of 0.1 N HCl were required for neutralization. Saponification number found, 499.

Hydrolysis of Fraction XIV.—This substance was hydrolyzed with 20 cc. of 5 per cent sulfuric acid as described above. During this operation the odor of acrolein was perceived, indicating that the material contained some decomposition products of glycerol which had been formed by the high temperature of the distillation. The acetic and sulfuric acids were removed in the manner described above and the remaining solution was

concentrated. The anhydrous residue was fractionated by distillation. A trace of substance came over at the boiling point of glycerol, then a small amount between 200–205°, at 0.1 mm. *This fraction analyzed for triglycerol.*

0.1072 gm. substance: 0.1852 gm. CO_2 and 0.0808 gm. H_2O.
$C_9H_{20}O_7$. Calculated. C 45.00, H 8.40.
Found. " 45.34, " 8.50.

Distillation Residue.—This material was a dark viscous mass which had a strong odor of acrolein, thus showing that some decomposition took place during distillation. The composition of the substance was the following.

0.1094 gm. substance: 0.2100 gm. CO_2 and 0.0678 gm. H_2O.
Found. C 52.34, H 6.93.

Saponification Number.—0.5522 gm. of substance was refluxed with 12 cc. of 0.5 N KOH for 3 hours. 19.9 cc. of 0.1 N HCl were required for neutralization. Saponification number found, 407.

Molecular Weight Determination by Method of Menzies and Wright.—30.7 cc. of benzene (b.p. 80.2°, at 760 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.5181	10	1095
1.0354	19	1150
1.7045	30	1200
2.3702	39	1280
2.8913	48	1275
3.1132	52	1265

Acetylation of Material.—10 gm. of this residue and 28 gm. of freshly distilled acetic anhydride were refluxed for 3 hours. The low boiling material was removed by distillation, first at reduced pressure (about 10 to 15 mm.) and subsequently at a pressure of 0.5 mm., at 230°. The residue analyzed as follows:

0.0988 gm. substance: 0.1908 gm. CO_2 and 0.0592 gm. H_2O.
Found. C 52.66, H 6.70.

Saponification Number.—0.2976 gm. of substance was refluxed with 10.1 cc. of 0.5 N KOH for 3 hours. 24.4 cc. of 0.1 N HCl were required for neutralization. Saponification number found, 493.

Molecular Weight Determination by Method of Menzies and Wright.—30.8 cc. of benzene (b.p. 79.6°, at 745 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
0.5214	10	1075
0.8218	16	1060
1.2828	23	1150
1.9014	36	1090
2.8051	53	1090
3.2452	61	1095

Hydrolysis of Residue.—To 31 gm. of the original residue were added 100 cc. of 5 per cent sulfuric acid and the solution was refluxed for 6 hours. The acetic and sulfuric acids were removed by the procedure described above. The hydrolyzed product could not be distilled at 0.5 mm. pressure although the outside temperature was raised to 230°. At this temperature the substance began to decompose. From the analysis and from the high boiling point of this product, and from the molecular weight determination of the acetate, it may be concluded that *the product, is made up of a long chain of glyceryl rests, perhaps not less than seven.* The analysis of the hydrolysate was as follows:

0.0903 gm. substance: 0.1585 gm. CO_2 and 0.0636 gm. H_2O.
$C_{21}H_{44}O_{15}$. Calculated. C 47.00, H 8.27.
Found. " 47.86, " 7.88.

Acetylation of This Hydrolyzed Residue.—To 10 gm. of the hydrolyzed residue were added 28 gm. of freshly distilled acetic anhydride and the mixture was refluxed for 3 hours. The product, which was worked up as described above, could not be distilled at 230°, at 0.2 to 0.4 mm. The analysis was as follows:

0.1003 gm. substance: 0.1887 gm. CO_2 and 0.0626 gm. H_2O.
$C_{39}H_{62}O_{24}$ (Nonoacetylhexaglycerylglycerol). Calculated. C 51.19, H 6.83.
Found. " 51.30, " 6.98.

Saponification Number.—0.3534 gm. of substance was refluxed with 10.05 cc. of 0.5 N KOH for 3 hours. 17.90 cc. of 0.1 N HCl were required for neutralization.

Saponification No. Calculated. 552. Found. 512.

Molecular Weight Determination by Method of Menzies and Wright.—29.8 cc. of benzene (b.p. 80.2° at 760 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	*mm.*	
1.3126	30	952
1.6647	38	953
2.1109	49	937
2.3965	56	931
2.8474	67	925

Molecular weight calculated, 915.

THE INFLUENCE OF THE INGESTION OF METHYL XANTHINES ON THE EXCRETION OF URIC ACID.*

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The early literature in the field of biochemistry contains the records of many investigations as to the effect of ingestion of methylated purines, particularly caffeine, on the excretion of uric acid, but the methods used were so inexact and the results obtained were so contradictory that very little dependence can be placed upon them. Some observers found that caffeine increased the uric acid of the urine, others found no change whatever in the uric acid excretion, but on the whole the greater weight of evidence perhaps favored the view that there is no increase in uric acid after the ingestion of caffeine.

About 10 years ago the development of more exact methods for the estimation of uric acid again caused attention to be directed to the effect of ingestion of methylated purines. In 1916 Benedict (1) and in 1917 Mendel and Wardell (2) published the records of investigations which seem to show quite conclusively that the ingestion of caffeine is followed by an increase in uric acid excretion and that this increase is perhaps caused by the demethylation and subsequent oxidation of at least a part of the ingested caffeine.

*The data here presented are taken in large part from a dissertation submitted by Emma L. Wardell to the Graduate College of the State University of Iowa, June, 1926, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

These observations were presented before the XIIth International Physiological Congress, Stockholm, Sweden, August 5, 1926 (*Skand. Arch. Physiol.*, 1926, xlix, 189).

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For some reason this newer view as to the effect of ingested caffeine does not yet appear to have received general acceptance. For instance, the most recent edition of Cushny's Pharmacology and Therapeutics[1] contains the statement that "uric acid of the urine is not increased by any of these drugs" (caffeine, theobromine, and theophylline). On this account it has seemed desirable to investigate further the effect of ingested methylated purines, not only caffeine, but also theobromine and theophylline.

The discovery of caffeine in coffee was made by Runge (3) in 1820 and a few years later theine was found in tea by Oudrey (4). Within another decade Jobst (5) proved the identity of theine and caffeine and Stenhouse (6) soon showed that caffeine is related to uric acid derivatives. Finally, Emil Fischer (7) proved its complete analogy to uric acid, and by preparing it from xanthine he showed it to be 1,3,7-trimethylxanthine, confirming the structure proposed by Medicus (8) in 1875. Fischer (9) also synthesized and proved the structure of theobromine, discovered in cocoa-beans by Woskrensensky (10) in 1842, and Fischer and Ach (11) later synthesized theophylline, which had been discovered in tea by Kossel (12) in 1888.

The wide-spread use of tea and coffee as beverages and the marked physiological effects following their ingestion and that of caffeine quite naturally led to investigations as to the fate of caffeine in the body. As early as 1850, Lehmann (13) reported that the administration of caffeine in the food was not followed by the excretion of even a trace of caffeine in the urine. Later experiments by many investigators show widely varying results, but on the whole they indicate that the ingestion of either coffee or caffeine is followed by the excretion of only a very small part of the ingested caffeine in the urine.

In the meantime the methylated purines of normal human urine, 1,7-dimethylxanthine, 7-monomethylxanthine, and 1-monomethylxanthine, had been isolated and carefully studied. Early experiments on the effect of caffeine ingestion on the excretion of the methylated purines show rather conflicting results, but perhaps the most striking are those of Albanese (14) in 1895 and of Bodzynski and Gottlieb (15) at about the same period. In experiments with dogs the former showed that caffeine feeding is followed by the appearance of a monomethylxanthine in the urine; the latter, in experiments with both dogs and rabbits, found similar results.

In animal experiments with theobromine and theophylline the results were much like those obtained with caffeine. Bodzynski and Gottlieb (15), Albanese (14), and Krüger and Schmidt (16) found that administration of theobromine to dogs was followed by the appearance of monomethylxanthine in the urine, and Krüger and Schmidt (17) also found that theophyl-

[1] Cushny, A. R., A text-book of pharmacology and therapeutics or the action of drugs in health and disease, Philadelphia and New York, 8th edition, 1924.

line had a comparable effect. Both Bodzynski and Gottlieb (15) and Krüger and Schmidt (16) performed similar experiments with theobromine on rabbits and obtained similar results.

About this time Krüger and Salomon (18) made a remarkable analysis of the purine base content of 10,000 liters of normal human urine from which they prepared 94.49 gm. of purine bases having the following composition:

	gm.
1,7–Dimethylxanthine	15.310
1-Methylxanthine	31.285
7-Methylxanthine	22.345
7-Methylguanine	8.400
Adenine	3.540
Hypoxanthine	8.500
Xanthine	10.110

Finally, with the aid of these invaluable analyses, Krüger and Schmidt (19) showed that since there is good evidence that in man the methyl groups are decreasingly stable in the order 7, 1, 3, the methyl purines of human urine can be easily accounted for by the removal from the methylated purines of the food of the methyl group occupying position 3.

Since demethylation is known to occur in the body, it seems not unlikely that a part of the ingested methylated purines may be completely demethylated and that the resulting purines may be subsequently oxidized to uric acid. Since much of the earlier work was on dogs it is of very little value, inasmuch as we now know that in the dog the chief end-product of purine metabolism is not uric acid but allantoin.

Perhaps the earliest report of the effect of coffee drinking on the excretion of uric acid in man is that of Leven (20) who, in 1868, found no increase at all. In 1899 Taylor (21) found an increase in uric acid after drinking coffee, but consideration of his experiments makes it very difficult to account for so large an increase in uric acid as the result of drinking so small a quantity of coffee if the transformation of the caffeine to uric acid is regarded as the sole cause of the increased uric acid excretion. Burian and Schur (22) found that the ingestion of caffeine had no effect on the quantity of uric acid in the urine, but Hess and Schmoll (23) found an increase after both tea and coffee drinking, and Haig (24) found similar results after the use of coffee. In all of these earlier investigations, however, the methods of estimating uric acid in the urine were not sufficiently exact to show accurately such small increases in uric acid excretion as might result from the partial transformation of ingested caffeine to uric acid.

The development of a colorimetric method made possible the more accurate estimation of small quantities of uric acid. In 1916, Benedict (1), using the Benedict-Hitchcock (25) modification of the Folin-Macallum-

Denis (26, 27) colorimetric procedure, secured very convincing evidence that the ingestion of caffeine does increase the excretion of uric acid. He reported a single experiment in which the subject was placed for a preliminary period of 3 days on an approximately constant purine-free diet, which included five cups of Kaffee Hag, a decaffeinated coffee. During the next period the diet was continued unchanged except that 200 mg. of caffeine were added to each cup of Kaffee Hag, making a total of 1 gm. of caffeine each day for 4 consecutive days. The caffeine was then discontinued but the purine-free diet was continued for 3 days longer. In this experiment it was found that during the ingestion of caffeine the uric acid excretion, measured by the Benedict-Hitchcock colorimetric method, rose gradually until on the 4th and last day it was more than 160 mg. above the endogenous level.

A few months later Mendel and Wardell, (2) using the same colorimetric procedure, found similar results with coffee, tea, and caffeine. Their subject remained throughout the investigations on a purine-free constant diet of milk, eggs, bread, butter, and fruit, to which was added in turn each of the substances to be studied. The results of these experiments seem to offer conclusive evidence that the ingestion of coffee, tea, or caffeine causes a marked increase in the excretion of uric acid and that the increase seems to be proportional to the quantity of caffeine ingested. It is interesting to note that the increase in the amount of uric acid excreted is equal to the quantity of uric acid which would be obtained by the demethylation and subsequent oxidation of from 10 to 15 per cent of the ingested caffeine.

More recently Clark and de Lorimier (28) have found that ingestion of either caffeine or theobromine tends to increase the concentration of uric acid in the blood, although the action of the former was much the more marked. Caffeine was found to increase the excretion of uric acid in the urine, but the initial increase was usually followed by a decreased excretion. Theobromine diminished the rate of excretion of uric acid although there seemed to be no change in its rate of formation. They conclude that the increase in uric acid excretion after the ingestion of caffeine is probably not due to direct oxidation of the methyl xanthines, and suggest that in the general metabolic stimulation, the renal cells first react to the increased endogenous uric acid causing an increased excretion, and that subsequently the excreting cells react to the foreign and more toxic substances (caffeine or various demethylated derivatives) resulting in a specific elimination of these substances to the partial exclusion of uric acid.

EXPERIMENTAL.

Since the reports in the older literature are very contradictory and many texts still state that the ingestion of methylated purines is without effect on the excretion of uric acid, it seemed desirable to study this problem again. In these new experiments we have studied the effect of ingestion of methyl xanthines on uric acid

excretion, using not only caffeine but also theobromine and theophylline and employing several methods for the estimation of uric acid.

In each experiment the subject was placed on a purine-free constant diet until the uric acid excretion reached its endogenous level. The methylated purine to be studied was then added to the diet for 3 or 4 days, and finally the purine-free diet was continued a few days longer.

The urine was collected in 24 hour periods. All laboratory determinations were done in duplicate. Total nitrogen was determined by the Kjeldahl method, purine base nitrogen by the Krüger-Schmidt method (29), and creatinine by the Folin method (30). Uric acid was determined by several different methods, and since the different procedures did not always yield comparable results, each method will be discussed in some detail.

Colorimetric Estimation of Uric Acid.—The Benedict-Hitchcock (25) modification of the Folin-Macallum-Denis procedure for estimation of uric acid in urine depends upon the fact that in alkaline solution uric acid is reduced by phosphotungstic acid with the formation of a deep blue color and that the intensity of the color is proportional to the amount of uric acid present. Since this reaction is also given by other substances present in the urine, the uric acid is first separated from these other substances by precipitating it as the silver salt. The silver salt is then dissolved and treated with the phosphotungstic acid reagent. Caffeine, theobromine, and theophylline were found not to react with the phosphotungstic reagent. No experiments were performed to determine the reaction of heteroxanthine, paraxanthine, or 1-methylxanthine with the reagent, but since the work of Lewis and Nicolet (31) indicates that none of the methyl xanthines causes a color development with the reagent, it was assumed that the presence of methyl xanthines in the urine offered no obstacle to the use of this method.

Throughout the course of these experiments the greatest care was taken to prevent deterioration of the standard solution of uric acid. New standard solutions were made at frequent intervals and carefully checked against the one then in use. Considerable difficulty was experienced with precipitation of the phosphates, and in some cases proportional quantities of the anhydrous diso-

dium hydrogen phosphate (Merck's Blue Label Reagent) and anhydrous potassium dihydrogen phosphate (Merck's Blue Label Reagent) were substituted for the crystallized salts specified by Benedict and Hitchcock.

The Benedict-Franke method of estimating uric acid, first published in 1922, makes possible an even more rapid and exact estimation of uric acid than could be obtained with the Benedict-Hitchcock procedure. In using the Benedict-Franke procedure (32), as with the Benedict-Hitchcock, the greatest care was taken to insure the absolute correctness of the standard solution. A new 50 mm. Bausch and Lomb Duboscq colorimeter was used for all colorimetric readings.

Estimation of Uric Acid and Purine Bases by Krüger-Schmidt Method.—In addition to the two colorimetric methods for estimating uric acid certain experiments were carried out in accordance with the Krüger-Schmidt (29) technique for estimating uric acid and purine bases. In this procedure, both uric acid and purine bases are precipitated in combination with copper oxide. This precipitate is then decomposed by sodium sulfide and the uric acid is next precipitated by means of hydrochloric acid. After filtration, the uric acid remaining in the filtrate is destroyed, the purine bases are precipitated from the filtrate by means of copper sulfate, and the nitrogen content of the precipitates of both uric acid and purine bases is determined by the Kjeldahl method.

Benedict and Saiki (33) report cases in which the total purine nitrogen as estimated by this method was less than the uric acid nitrogen as estimated by the Folin-Shaffer method, and find that the inaccuracy lies in the Krüger-Schmidt method. For this reason they advise the addition of 20 cc. of glacial acetic acid for each 300 cc. of urine, the acid to be added before the first precipitation. This precaution was observed in our experiments. On the whole, however, the Krüger-Schmidt procedure was found to be unsatisfactory for the estimation of uric acid, although no difficulty was experienced in the estimation of purine bases.

Gravimetric Estimation of Uric Acid.—In order to check further the accuracy of the colorimetric estimations, an effort was made to isolate and weigh the uric acid from a given amount of urine. This was done following a suggestion made to us by Dr. S. R. Benedict. In 1915 Benedict (34) successfully employed such a

procedure as a check on the combined uric acid which he had found in ox blood by the colorimetric method after acid hydrolysis. Gravimetric determinations of the uric acid were carried out in one experiment (see Table VIII), after sufficient technique had been acquired by running through a considerable number of isolations. Although the gravimetric method is quite tedious and requires much care in manipulation, it is possible to obtain very satisfactory results. In most instances the checks with the Benedict-Franke method have been surprisingly close, considering the difficulties of quantitatively isolating the uric acid. We believe that the figures recorded in Table VIII are a sufficient proof of this statement, although three times as many observations might be given. If these analyses have done nothing else, they have substantiated the accuracy of the Benedict-Franke colorimetric method. The technique we have employed is given below.

(*a*) *Isolation.*—Place as much urine as contains 5 to 7 mg. of uric acid (usually 25 cc.) in a 50 cc. centrifuge tube, add 5 cc. of ammoniacal silver-magnesium solution and 3 cc. of concentrated ammonia (to dissolve the silver chloride). Stir and centrifuge for 5 minutes. Pour off the supernatant fluid, remove ammonia as completely as possible with compressed air, and stir up the precipitate with 5 cc. of water. Add 5 cc. of freshly saturated hydrogen sulfide water and stir thoroughly for a few minutes. Wash into a beaker, remove the hydrogen sulfide by boiling, and the silver sulfide by filtration. Boil the filtrate to small volume (approximately 10 cc.), transfer to a 50 cc. centrifuge tube, and cool. Add 5 cc. of ammoniacal silver-magnesium solution, stir, and centrifuge. Treat the precipitate with hydrogen sulfide water as before, boil, filter, and concentrate to 10 cc. Transfer to a small beaker and evaporate to 5 cc. on a water bath. Remove from the water bath and add *very slowly* 2 cc. of dilute hydrochloric acid (1 part of acid to 9 parts of water, by volume). Allow to stand (uncovered) until the uric acid has crystallized (40 to 60 hours). Wash the crystals into a dry, weighed, 15 cc. centrifuge tube and centrifuge for 3 minutes. Wash twice with a little water and once with alcohol. Dry to constant weight at 90°.

(*b*) *Identification.*—Dissolve the precipitate in 15 cc. of hot phosphate solution (500 cc. of phosphate solution contain 1 gm. of crystallized NaH_2PO_4 and 9 gm. of crystallized Na_2HPO_4, or

corresponding quantities of the anhydrous compounds). Use 10 cc. of this solution for a nitrogen estimation and 3 cc. for a colorimetric estimation.

(*c*) *Nitrogen Determinations.*—Use regular Kjeldahl procedure, distilling into 0.02 N acid and titrating with 0.02 N alkali. If duplicate precipitates are available, one may be dissolved directly

TABLE I.

Effect of Ingestion of Caffeine on Excretion of Uric Acid.

Subject W., female; age 38 years, weight 62.2 kilos.

Date.	Composition of urine.					Remarks on diet.
	Volume.	Total N.	Creatinine.	Uric acid (Benedict-Franke).	Purine base N.	
Nov., 1926	*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	
22	1230	8.8	0.99	315	14	Purine-free.*
23	975	9.2	0.98	305	15	"
24	860	10.3	0.99	261	11	"
25	1485	9.6	1.03	426	52	Purine-free + 0.94 gm. caffeine.
26	875	9.9	1.04	504	76	" + 1 " "
27	970	9.5	0.96	475	73	" + 1 " "
28	720	10.3	0.98	357	30	Purine-free.
29	975	9.8	0.95	247	13	"
30	1215	10.2	0.98	241	10	"

* Milk, eggs, grapefruit.

with concentrated sulfuric acid and transferred quantitatively to a Kjeldahl flask for nitrogen determination.

(*d*) *Benedict-Franke Colorimetric Estimation.*—Place 3 cc. of the solution (containing one-fifth of the precipitate) in a 50 cc. volumetric flask, add about 15 cc. of water and 2.5 cc. of dilute hydrochloric acid (1 part of acid to 9 parts of water), dilute to the mark, and mix. To develop color, place 10 cc. of this solution in a 50 cc. volumetric flask, add 5 cc. of 5 per cent sodium cyanide

solution and 1 cc. of the arsenophosphotungstic acid reagent, and mix. Allow to stand for 5 minutes, dilute to the mark, and compare with 0.2 mg. standard solution similarly developed.

Experiments with Caffeine.—Four experiments with caffeine were carried out on four different subjects. The results of these experiments are found in Tables I to IV. A study of these figures shows that in two cases (see Tables I and II) the uric acid

TABLE II.

Effect of Ingestion of Caffeine on Excretion of Uric Acid.

Subject K., female; age 25 years, weight 42.7 kilos.

Date.	Composition of urine.						Remarks on diet.
				Uric acid.			
	Volume.	Total N.	Creatinine.	Benedict-Hitchcock.	Benedict-Franke.	Purine base N.	
Jan., 1926	*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	
7	520	7.4	0.76	247	230	10	Purine-free.*
8	505	7.9	0.75	223	213	8	"
9	460	7.1	0.75	235	221	12	"
10	1020	8.4	0.69	326	321	26	Purine-free + 1 gm. caffeine.
11	490	6.4	0.71	351	341	28	" + 1 " "
12	515	7.1	0.71	372	356	33	" + 1 " "
13	580	7.2	0.74	354	331	47	" + 0.786 " "
14	220	6.9	0.76	290	271	30	Purine-free.
15	355	7.5	0.73	258	250	22	"
16	565	8.1	0.74	257	245	9	"
17	540	7.5	0.69	239	226	14	"

* Milk, eggs, crackers, grapefruit.

excretion as estimated by the new Benedict-Franke method was markedly increased during the period of caffeine ingestion and returned to its endogenous level within 2 or 3 days after the discontinuance of the caffeine. With the other two subjects there was a marked rise in uric acid excretion on the 1st day of caffeine ingestion. After the 1st day, however, the uric acid values gradually became less until on the last day of caffeine

ingestion, the uric acid was back practically to its endogenous level where it remained relatively constant throughout the final period.

An examination of Table IV shows a marked rise in the uric acid on the 2nd day of the final purine-free period. A similar inexplicable rise in uric acid while on a purine-free diet had been previously encountered in the same subject. The subject is a

TABLE III.

Effect of Ingestion of Caffeine on Excretion of Uric Acid.

Subject M., male; age 43 years, weight 79.5 kilos.

Date.	Composition of urine.					Remarks on diet.
	Volume.	Total N.	Creatinine.	Uric acid (Benedict-Franke).	Purine base N.	
Mar., 1926	*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	
1	730	10.2	1.50	474		Purine-free.*
2	710	10.6	1.62	450	11	"
3	740	10.4	1.62	417	10	"
4	630	10.7	1.62	421	11	"
5	1020	12.7	1.56	495	36	Purine-free + 1.5 gm. caffeine.
6	655	10.0	1.59	486	88	" + 1.5 " "
7	850	10.9	1.65	450	97	" + 1.5 " "
8	590	11.7	1.62	411		" + 1.5 " "
9	640	11.3	1.62	391	54	Purine-free.
10	835	12.1	1.59	413		"
11	815	12.1	1.62	375	10	"

* Shredded Wheat, milk, eggs, bread, butter, potatoes, banana, orange, sugar, ice-cream.

normal healthy young woman doing routine laboratory work. No drugs, other than caffeine, were used during the experiment. In both experiments every precaution was taken to insure rigid adherence to diet, and it seems certain that none of these factors is responsible for the unexpected increase in uric acid on a purine-free diet.

Experiments with Theobromine.—Experiments on the effect of ingestion of theobromine were carried out simultaneously on two subjects (see Tables V and VI). In neither case was there any

TABLE IV.

Effect of Ingestion of Caffeine on Excretion of Uric Acid.

Subject H., female; age 25 years, weight 52.1 kilos.

Date.	Composition of urine.				Remarks on diet.
	Volume.	Total N.	Creatinine.	Uric acid (Benedict-Franke).	
Mar., 1916	*cc.*	*gm.*	*gm.*	*mg.*	
4	740	10.5	1.05	400	Purine-free.*
5	1545	12.9	1.18	407	"
6†					"
7	910	11.6	1.08	404	"
8	1315	12.6	1.14	420	"
9	1250	12.3	1.15	424	"
10	2220	13.0	1.17	646	Purine-free + 1.2 gm. caffeine daily.
11‡	1725	12.8	1.26	621	" + 1.2 " " "
12§	1750	12.2	1.07	556	" + 1.2 " " "
13	1460	12.4	1.18	505	" + 1.2 " " "
14	1490	12.1	1.18	489	" + 1.2 " " "
15	1015	12.6	1.09	395	Purine-free.
16	1450	12.0	1.17	484	"
17†					"

* Milk, eggs, bread, rusks, butter, cauliflower, lettuce, salad oil, grapefruit, sugar.

† Specimen lost.

‡ 27 hour specimen.

§ 21 hour specimen.

increase in uric acid excretion accompanying the ingestion of 2 gm. of theobromine daily for 4 consecutive days. Since this failure to find an increase in uric acid was somewhat unexpected, it seemed well to try the effect not only of the Merck product first used,

but also some prewar Kahlbaum's theobromine. As Tables V and VI show, the ingestion of the Kahlbaum preparation had no more effect on uric acid excretion than the other. The nitrogen content of the Merck theobromine was found to be 30.87 per cent. Since the theoretical value is 31.10 per cent, it seems fair to conclude that the failure to secure an increase in uric acid excretion was not due to any fault of the theobromine. Furthermore, it

TABLE V.

Effect of Ingestion of Theobromine on Excretion of Uric Acid.

Subject W., female; age 38 years, weight 61.3 kilos.

Date.	Composition of urine.					Remarks on diet.
	Volume.	Total N.	Creatinine.	Uric acid (Benedict-Franke).	Purine base N.	
Feb., 1920	*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	
9	760	5.5	1.04	238	16	Purine-free.*
10	1030	6.3	1.00	238	13	"
11	1485	7.4	1.04	230	11	"
12	1110	7.4	1.00	252	85	Purine-free + 2 gm. theobromine (Merck).
13	1100	8.5	0.99	220	98	" + 2 " " "
14	1210	7.0	1.05	220	113	" + 2 " " " (Kahlbaum).
15	890	7.6	0.98	212	130	" + 2 " " " "
16	755	6.5	0.97	239	111	Purine-free.
17	1070	7.2	0.98	242	28	"

* Milk, Corn Flakes, crackers, butter, orange, apple.

could scarcely have been due to a failure of absorption since the ingestion of theobromine was accompanied by a large increase in the excretion of purine bases. Clark and de Lorimier (28) likewise failed to detect any augmenting influence of theobromine on uric acid excretion.

Experiments with Theophylline.—Three experiments were performed with theophylline. In these experiments the subjects

did not ingest theophylline itself, but euphyllin, which contains 78 per cent theophylline and 22 per cent ethylenediamine. This preparation has the double advantage of being readily soluble and much less irritant than theophylline alone. It was obtained in tablets, of which a specified number was taken each day.

The results of the first experiment are shown in Table VII. On the 1st day of euphyllin ingestion the increase in uric acid ex-

TABLE VI.

Effect of Ingestion of Theobromine on Excretion of Uric Acid.

Subject K., female; age 25 years, weight 42.7 kilos.

Date.	Composition of urine.				Remarks on diet.
	Volume.	Total N.	Creatinine.	Uric acid (Benedict-Franke).	
Feb., 1916	*cc.*	*gm.*	*gm.*	*mg.*	
9	730	4.8	0.76	212	Purine-free.*
10	680	6.4	0.78	201	"
11	720	6.4	0.76	188	"
12	490	6.4	0.77	182	Purine-free + 1.25 gm. theobromine (Merck).
13	700	5.8	0.73	176	" + 1.25 " " "
14	375	5.1	0.77	170	" + 1.25 " " (Kahlbaum).
15	620	5.4	0.74	176	" + 1.25 " " "
16	405	4.9	0.75	199	Purine-free.
17	675	5.7	0.77	212	"

* Milk, Corn Flakes, crackers, grapefruit.

cretion as measured by the Benedict-Franke method was much greater than the increase as measured by the Benedict-Hitchcock method. On succeeding days the Benedict-Franke values gradually became less and the Benedict-Hitchcock values increased until on the 3rd and 4th days the results obtained by the two procedures were practically equal at a point well above the endogenous level.

With the discontinuance of euphyllin the excretion of uric acid dropped back to its endogenous level within 24 hours.

So unexpected was the discrepancy between the values obtained on the 1st euphyllin day by the two colorimetric procedures that every effort was made to leave no doubt as to the correctness of the figures. The estimations were repeated immediately with the same results. New standards were prepared at once and the

TABLE VII.

Effect of Ingestion of Euphyllin on Excretion of Uric Acid.

Subject W., female; age 38 years, weight 60 kilos.

Date.	Fluid intake.	Composition of urine.				Remarks on diet.
		Volume.	Creatinine.	Uric acid.		
				Benedict-Hitchcock.	Benedict-Franke.	
Apr., 1926	*cc.*	*cc.*	*gm.*	*mg.*	*mg.*	
20	1550	1200	1.02	327	318	Purine-free.*
21	1550	1000	1.02	266	246	"
22	1550	1295	0.98	260	237	"
23	2050	1750	1.01	287	357	Purine-free + 360 mg. euphyllin.
24	2050	1745	0.97	306	339	" + 360 " "
25	2050	1830	0.99	324	331	" + 360 " "
26	2400	2150	1.02	314	311	" + 360 " "
27	1550	1260	1.03	259	238	Purine-free.
28	1550	1275	1.00	260	241	"

* Milk, graham crackers, peanut butter, sugar cookies, orange, apple.

estimations were repeated, the new standards being used, but the same values were obtained. These estimations were all made early in the morning. Late in the day, however, the estimations were made again and this time the Benedict-Hitchcock method still gave practically the same value, 285 mg., but the Benedict-Franke gave only 296 mg.

This change in value as estimated by the Benedict-Franke method was so striking that the next day the estimations by both

methods were repeated at intervals throughout the day. The results were as follows:

	Benedict-Hitchcock.	Benedict-Franke.
	mg.	*mg.*
Estimations made at 10.00 a.m.....	306	339
" " " 12.30 p.m.....	305	320
" " " 2.00 "	309	308
" " " 5.00 "	304	286

These figures seem to indicate that there was present in the urine some substance other than uric acid which reacted with the Benedict-Franke reagent and which lost that power of reaction on standing. This phase of the results of the euphyllin experiment will be discussed again.

In passing, it seems worth while to call attention to the fact that the ingestion of less than 300 mg. of theophylline was accompanied by an increase of 100 mg. in uric acid excretion, a quantity which would represent the transformation of 33 per cent of the theophylline ingested.

This experiment with theophylline was the only one in which a a careful record of fluid intake was kept. A comparison of the figures for fluid intake and for urine volume shows plainly that what seems to be an increased volume of urine during the euphyllin period is not really a diuresis because the increase in fluid intake was quite equal to the increase in urine volume.

A later experiment on the same subject (see Table VIII) shows only a slight increase in uric acid excretion following the ingestion of euphyllin, but a third experiment on another subject shows a considerable increase in uric acid excretion. In this last experiment, the subject ingested 500 mg. of euphyllin, containing 390 mg. of theophylline, each day for 3 consecutive days. On the 1st day of euphyllin ingestion, the excretion of uric acid was only slightly greater than during the preliminary period, but on the 2nd and 3rd days of the euphyllin period it was very greatly increased.

In both of these later experiments the uric acid was estimated by both the Benedict-Franke and Benedict-Hitchcock procedures and throughout the experiments the values obtained by the Benedict-Hitchcock procedure were slightly higher than those obtained by the Benedict-Franke method. In neither case did

the ingestion of euphyllin cause a reversal in the relative values of the two estimations of uric acid similar to that found in the first theophylline experiment.

In the second experiment with euphyllin recorded in Table VIII

TABLE VIII.

Effect of Ingestion of Euphyllin on Excretion of Uric Acid, Including Comparison of Colorimetric and Gravimetric Figures for Uric Acid.

Subject W., female; age 39 years, weight 61 kilos.

Date.	Volume.	Total N.	Creatinine.	Uric acid.			Estimations on precipitate.				Diet.
				Benedict-Hitchcock.	Benedict-Franke.	Gravimetric.	Weight.	Benedict-Franke.	N (theory).	N (found).	
June, 1927	*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
								5.90*			
6	1490	6.1	0.93	277	253	249	5.8	**5.95**	1.93	1.89	Purine-free.†
								5.80			
7	1520	6.1	0.91	274	252	243	5.6	**5.81**	1.87	1.77	"
								8.05			
8	830	6.6	0.91	247	230	232	8.4	**8.42**	2.80	2.82	"
								6.72			
9	1470	7.7	0.99	315	288	273	6.5	**6.24**	2.17	2.06	Purine-free + 500 mg. euphyllin.
								7.44			
10	1190	8.2	0.94	280	255	292	8.6		2.87	2.82	" "
								7.30			
11	1270	8.6	0.92	292	271	268	7.4	**7.02**	2.47		" "
								6.84			
12	1350	7.7	0.91	291	264	258	6.7	**6.42**	2.23		Purine-free.
								6.94			
13	1290	6.4	0.93	263	247	251	6.8	**6.50**	2.27	2.36	"

* Figures set in ordinary type were obtained by direct estimation on urine. Those in bold faced type were obtained by Benedict-Franke estimation on the isolated uric acid.

† Milk, biscuit, crackers, sugar cookies, lemons.

uric acid was determined gravimetrically as well as by the two Benedict colorimetric methods. It will be noted that during the two control periods the gravimetric figures excellently check those obtained by the Benedict-Franke method, but that on the 2nd

euphyllin day, the gravimetric result is 37 mg. higher, possibly due to interference of 1-methylxanthine. Too great significance should not therefore be attached to this difference.

Of all our experiments, the second and third experiments with euphyllin show most strikingly the stimulation of metabolism which follows the use of these drugs. The subject of the second experiment showed a marked increase in total nitrogen excretion

TABLE IX.

Effect of Ingestion of Euphyllin on Excretion of Uric Acid.

Subject H., male; age 31 years, weight 70 kilos.

Date.	Composition of urine.					Remarks on diet.
				Uric acid.		
	Volume.	Total N.	Creatinine.	Benedict-Hitchcock.	Benedict-Franke.	
June, 1927	*cc.*	*gm.*	*gm.*	*mg.*	*mg.*	
13	1170	7.5	1.86	260	249	Purine-free.*
14	970	7.3	1.60	289	265	"
15	750	7.8	1.04	263	227	"
16	1300	10.8	1.05	306	273	Purine-free + 500 mg. euphyllin.
17	1870	16.0	1.89	510	468	" + 500 " "
18	1880	12.4	1.54	404	396	" + 500 " "
19	1350	10.1	1.30	329	294	Purine-free.
20	1560	8.2	1.28	346	328	"
			1.08	293	269	"

* Milk, eggs, oatmeal, toast, butter, potatoes, tomatoes, lettuce, spinach, orange, lemon.

although the increase in uric acid was very slight. In the third experiment, however, there was an enormous increase not only in uric acid, but also in total nitrogen and in creatinine.

Experiments with Methyl Uric Acids.—The results of some of the experiments on caffeine suggest the possibility that caffeine ingestion is followed by the presence in the urine of some product of metabolism other than uric acid which also reacts with the reagents of the Benedict-Hitchcock and Benedict-Franke colorimetric methods. As has already been stated, a small part of the caffeine

ingested may appear in the urine unchanged, but a far larger portion of it is partly demethylated in the process of metabolism and appears in the urine as 7-methylxanthine, 1-methylxanthine, and 1,7-dimethylxanthine (18). The possibility that the apparent increase in uric acid was really caused by the presence of caffeine in the urine is easily ruled out, since neither caffeine, theobromine, nor theophylline was found to give any reaction with either of the Benedict reagents. Moreover, the work of Funk and Macallum (35) and of Lewis and Nicolet (31) shows that neither xanthine, hypoxanthine, nor 1,7-dimethylxanthine gives any reaction with either of the reagents. No data have been found in regard to 1-methylxanthine, but since Lewis and Nicolet report negative results with twelve different methylated monooxypurines and dioxypurines, it seems reasonable to assume that 1-methyl-2,6-dioxypurine and 7-methyl-2,6-dioxypurine would also give negative results.

The methylated purines, however, are present in the urine in quantity much too small to account for all the caffeine ingested. There remains, then, the possibility that some of the ingested caffeine is oxidized in the 8 position before it is completely demethylated, thus forming methyl uric acids. Assuming that such oxidation does take place there might be formed in the processes of metabolism such compounds as 7-methyluric acid, 1-methyluric acid, 1,7-dimethyluric acid, 3-methyluric acid, 3,7-dimethyluric acid, 1,3-dimethyluric acid, and 1,3,7-trimethyluric acid. Since, however, demethylation is most likely to take place in the order 3,1,7, it is probable that the compounds most likely to be formed would be 7-methyluric acid, 1-methyluric acid, and 1,7-dimethyluric acid.

None of the methylated uric acids has ever been found in the urine but all of them have been synthesized and their properties have been studied by a number of investigators. The earliest work seems to be that of Hill (36) and of Hill and Mabery (37) who first prepared methyl- and dimethyluric acid by heating lead urate with methyl iodide in a closed tube. Most of our information in regard to these compounds, however, is the result of the painstaking work of E. Fischer (38) and of Biltz (39), who synthesized the various possible methyl uric acids and studied their properties.

XUM

Our present study of these compounds was made possible through the kindness of Professor Biltz of the University of Breslau who supplied us with small quantities of 7-methyluric acid, 3,7-dimethyluric acid, and 1,3-dimethyluric acid. More recently he has supplied us with larger quantities of 1-methyluric acid, 1,3-dimethyluric acid, 3,7-dimethyluric acid, 3,9-dimethyluric acid, and 1,3,7-trimethyluric acid. We have thus had the opportunity of examining all the interesting uric acids except 3-methyluric acid and 1,7-dimethyluric acid.

In testing each of these compounds with the Benedict-Franke procedure, a solution of the methyl uric acid was made in exactly the same way as was the strong standard uric acid solution. 5 cc. of this solution were then placed in a 50 cc. Erlenmeyer flask, 5 cc. of a 5 per cent solution of sodium cyanide and 1 cc. of the arsenophosphotungstic acid reagent were added, and the contents of the flask were gently mixed. At the end of 5 minutes the mixture was diluted to 50 cc. and compared in the colorimeter with a standard uric acid solution prepared at the same time and in exactly the same way. With this procedure both the 7-methyluric acid and the 3,7-dimethyluric acid gave a mere trace of color, while 1,3,7-trimethyluric acid gave about 5 per cent of the color development of uric acid and 3,9-dimethyluric acid about 15 per cent. On the other hand, 1-methyluric acid and 1,3-dimethyluric acid gave approximately the same color development as uric acid when compared on a gm. molecule basis.

Similar experiments were performed in which aqueous suspensions of the pure compounds were used instead of first dissolving them in the phosphate solutions. The results obtained in this way were similar to those of the first experiment; neither 7-methyluric acid nor 3,7-dimethyluric acid gave more than a mere trace of color, 1,3,7-trimethyluric acid gave a little more color, while 1-methyluric acid and 1,3-dimethyluric acid gave a color development approximately equal to that of uric acid.

Methyl uric acids are unstable in alkali, particularly hot alkali, and it was found that the temperature of the phosphate mixture in which the uric acid was dissolved had a direct bearing on the amount of color which the solution developed when treated with the reagent. When a cold alkaline phosphate was employed to

dissolve the methyl uric acids, before adding acetic acid, a definitely greater color development was obtained.

The color-forming value of the phosphate solution of each of these methyl uric acids was then tested by the Benedict-Hitchcock procedure. In each case, 5 cc. of the phosphate solution were precipitated with 20 drops of the ammoniacal silver-magnesium solution and centrifuged for 2 minutes. The supernatant fluid was poured off and the color developed in accordance with the regular Benedict-Hitchcock technique. The color was then compared with that obtained by simultaneously treating an equal quantity of the standard uric acid solution in exactly the same way. The results of this experiment proved interesting, but rather damaging to the methyl uric acid hypothesis. Both 7-methyluric acid and 3,7-dimethyluric acid gave just a trace of color with this procedure, but 1-methyluric acid and 1,3-dimethyluric acid gave no color at all. It is further of interest that whereas without silver precipitation 1-methyluric acid gave almost as great a color development with the Benedict-Hitchcock reagents as with the Benedict-Franke reagents, 1,3-dimethyluric acid gave only about half as much color as pure uric acid with the Benedict-Hitchcock reagents.

In order to be sure that any 1,3-dimethyluric acid possibly present in urine does not respond to the Benedict-Hitchcock procedure, the following experiment was performed. 2 cc. of urine were placed in each of two centrifuge tubes, to one of which 5 cc. of a phosphate solution of 1,3-dimethyluric acid (containing 1 mg. of the acid) were added, and the contents of both tubes were then submitted to the Benedict-Hitchcock procedure for estimating uric acid. The color developed in the two solutions was identical, being equal to that produced by 0.68 mg. of uric acid. Obviously any 1,3-dimethyluric acid that may be present in the urine will not respond to the Benedict-Hitchcock procedure for estimating uric acid. Apparently the compound is decomposed after being precipitated as the silver salt in ammoniacal solution.

DISCUSSION.

Although the investigators who have recently studied this subject (Benedict (1), Mendel and Wardell (2), Clark and de Lorimier (28), and the writers) agree that the ingestion of certain methyl xanthines, notably caffeine, gives rise to an increased ex-

cretion of uric acid as determined by the colorimetric method, the source of this extra uric acid does not appear to have been conclusively proved.

There are four possible explanations for this increased excretion of uric acid following the ingestion of certain methyl xanthines: (1) *increased cellular metabolism*, (2) *increased activity of the kidneys*, (3) *demethylation of the methyl xanthines and subsequent conversion to uric acid*, and (4) *oxidation of the methyl xanthines in position 8 without demethylation or with only partial demethylation*, thus giving rise to the presence of certain methyl uric acids which give the uric acid color reaction. Evidence is unavailable at the present time conclusively proving any of these views.

As has been shown by Means, Aub, and Du Bois (40) and others caffeine will augment the basal metabolism from 7 to 23 per cent. The factor of increased cellular activity is therefore one that cannot be ignored, and is the explanation to which apparently Clark and de Lorimier (28) particularly lean to account for the increased excretion of uric acid which they observed after caffeine. We do not regard this as a sufficient explanation of the markedly augmented elimination of uric acid noted in some individuals after caffeine and theophylline, although it may well play a part. As shown in our own experiments, the nitrogen output is not ordinarily much disturbed, although in the last experiment (Table IX) with theophylline the nitrogen output was considerably increased.

The diuretic action of the methyl xanthines under certain conditions is well known, and it has been recognized for a number of years that salicylic acid, cinchophen, and many of their derivatives (41) have a peculiar stimulating influence on uric acid excretion. For several reasons we do not believe that such a stimulating effect on the kidney is an important factor in the action of some of the methyl xanthines. Theobromine has been more widely used on account of its diuretic action than either caffeine or theophylline, and still it has no augmenting influence on uric acid elimination according to the observations of Clark and de Lorimier (28) and ourselves. In the case of cinchophen, salicylic acid, and their derivatives it is well known that the increased uric acid excretion is accompanied by a reduction of the blood uric acid to a very low level (41), but the observations of Clark and de Lorimier show a considerable rise in the blood uric acid after caffeine ingestion.

XUM

It is known that about 50 per cent of ingested xanthine is excreted by the human subject in the form of uric acid (42). We possess very good evidence that, when the methyl xanthines are taken by man, they are at least partly demethylated. It is perfectly reasonable to assume therefore that some of these methyl xanthines should be completely demethylated, and thus give rise to xanthine and ultimately in part to uric acid. We are inclined to believe that this is the explanation for a part of the increased uric acid excretion encountered after caffeine.

In looking around for an explanation as to why many of the older workers came to the conclusion that caffeine did not increase the excretion of uric acid, whereas recent investigators who have employed the new colorimetric methods have obtained positive results, it occurred to us that this discrepancy might be due to the presence in the urine of certain methyl uric acids which would give the color reactions for uric acid, but possess sufficiently different physical properties so that they would not be determined by the older methods. One question which might at once be raised is, why, if methyl uric acids are excreted in the urine after the ingestion of methyl xanthines, have they not already been detected in the urine? These compounds are so unstable that they would probably have been decomposed by the customary methods of isolation. As soon as we were able to obtain some of the methyl uric acids we observed a number of facts of an interesting circumstantial character. For example on this basis it is easy to explain why theobromine does not lead to an increased excretion of uric acid as determined by the colorimetric method, and why theophylline does lead to such an increased excretion. The most damaging evidence we have obtained against this hypothesis is that the two methyl uric acids (1- and 1,3-) which we have found to give approximately the same color development as uric acid with the Benedict-Franke method are decomposed by the silver precipitation of the Benedict-Hitchcock method. Our observations on the pure methyl uric acids will be discussed in light of the results obtained in the experiments with ingested caffeine, theobromine, and theophylline.

Since theobromine is 3,7-dimethylxanthine, the only possible methyl uric acids which could be formed from it are 3-methyluric acid, 7-methyluric acid, and 3,7-dimethyluric acid. Of these,

the last two have been proved to give only a trace of color with either of the Benedict reagents. It is true that Funk and Macallum (35) report that 3-methyluric acid gives a positive reaction with the phosphotungstic reagent, but they say nothing in regard to the intensity of the color developed nor do they mention the degree of stability of the silver salt when precipitated in ammoniacal solution. Moreover, the methyl group in position 3 is the one generally regarded as most easily lost during metabolism.

In the experiments on the ingestion of theobromine, there was no increase whatever in the excretion of uric acid as estimated by the Benedict-Franke colorimetric method. Obviously, then, none of it was completely demethylated and subsequently oxidized to uric acid, but, on the other hand it is not at all impossible that some of it was oxidized before demethylation, since neither 7-methyluric acid nor 3,7-dimethyluric acid would increase the value of the uric acid as estimated by either of the colorimetric procedures.

The results of the first theophylline experiment are even more interesting when examined in this connection, since there are several theoretically possible demethylation-oxidation products. The theophylline may be completely demethylated and subsequently oxidized to uric acid, causing an increase in the uric acid excretion as estimated by both of the colorimetric methods, or it may be oxidized previous to demethylation with formation of 1-methyluric acid, 3-methyluric acid, or 1,3-dimethyluric acid. The action of 3-methyluric acid when tested with the colorimetric methods has not been covered in these studies, but if 1-methyl- or 1,3-dimethyluric acid were formed during metabolism, there would be an increase in uric acid in the urine as estimated by the Benedict-Franke procedure but not as estimated by the Benedict-Hitchcock method.

As a matter of fact, in this experiment the ingestion of theophylline caused an increase in uric acid excretion which was far greater when estimated by the Benedict-Franke method than with the Benedict-Hitchcock method. If we assume that man can oxidize theophylline without first demethylating it, this apparent discrepancy in the estimation of uric acid might very well be due to the presence in the urine of 1-methyl- or 1,3-dimethyluric acid. The steady decline of the Benedict-Franke values could then be

explained by the gradually decreasing excretion of 1-methyl- or 1,3-dimethyluric acid and the corresponding increase in the Benedict-Hitchcock values could be attributed to a corresponding increase in uric acid, or possibly 3-methyluric acid.

In the experiments with caffeine there was always a marked increase in uric acid excretion estimated by either of the colorimetric methods. This increase may be due in part to a stimulation of metabolism or it may be due to the formation of uric acid by the total demethylation and subsequent oxidation of the caffeine. On the other hand, it is theoretically possible that the increase is due, in whole or in part, to the presence of a methyl uric acid which responds to both color tests. We have not yet had the opportunity of testing 3-methyluric acid or 1,7-dimethyluric acid.

Experiments are being continued in the hope of more fully elucidating this problem.

SUMMARY.

The ingestion of caffeine is followed by an unmistakable increase in the excretion of uric acid. This increase is practically the same when estimations are made by either the Benedict-Hitchcock or Benedict-Franke procedures. The ingestion of theobromine is not followed by an increase in the excretion of uric acid, but the ingestion of theophylline is usually followed by a definite increase.

The accuracy of the Benedict-Franke colorimetric method has been checked with gravimetric estimations of the uric acid and excellent agreement obtained.

At least two methyl uric acids (1-methyluric acid and 1,3-dimethyluric acid) give the color reaction with the Benedict-Franke procedure to the same degree as does uric acid itself, but fail to give any reaction whatever with the Benedict-Hitchcock procedure. Other methyl uric acids, notably 7-methyluric acid and 3,7-dimethyluric acid, fail to give a color with either of the colorimetric procedures, while 1,3,7-trimethyluric acid gives only a trace of color.

It is an interesting fact of circumstantial character that whereas 3,7-dimethylxanthine (theobromine) does not cause an increase in the excretion of uric acid as determined colorimetrically and

3,7-dimethyluric acid does not give the color reaction, on the other hand 1,3-dimethylxanthine (theophylline) does increase the output of uric acid and 1,3-dimethyluric acid does give the color reaction. It is furthermore significant that 1,3-dimethylxanthine (theophylline) causes a greater increase in uric acid excretion (first experiment) when estimated by the Benedict-Franke procedure than when estimated by the Benedict-Hitchcock method and that 1,3-dimethyluric acid does respond to the Benedict-Franke procedure but not to the Benedict-Hitchcock method.

The origin of the increase in uric acid excretion observed after caffeine and theophylline has not been definitely ascertained. Stimulation of metabolism alone seems insufficient to account for all the extra uric acid, and it is suggested that the increase may be due, at least in part, to the transformation of caffeine and theophylline to uric acid or methyl uric acids.

BIBLIOGRAPHY.

1. Benedict, S. R., *J. Lab. and Clin. Med.*, 1916, ii, 1.
2. Mendel, L. B., and Wardell, E. L., *J. Am. Med. Assn.*, 1917, lxviii, 1805.
3. Runge, Phytochemische Entdeckungen, Berlin, 1820; cited by McCrudden, F. H., Uric acid, New York, 1905.
4. Oudrey, *Mag. Pharm.*, 1827, xix, 49.
5. Jobst, C., *Ann. Chem.*, 1838, xxv, 63.
6. Stenhouse, J., *Ann. Chem.*, 1843, xlv, 366.
7. Fischer, E., *Ber. chem. Ges.*, 1881, xiv, 637, 1905.
8. Medicus, L., *Ann. Chem.*, 1875, clxxv, 230.
9. Fischer, E., *Ber. chem. Ges.*, 1882, xv, 453; 1897, xxx, 1839.
10. Woskrensensky, A., *Ann. Chem.*, 1842, xli, 125.
11. Fischer, E., and Ach, L., *Ber. chem. Ges.*, 1895, xxviii, 2473, 3135.
12. Kossel, A., *Ber. chem. Ges.*, 1888, xxi, 2164; *Z. physiol. Chem.*, 1889, xiii, 298.
13. Lehmann, C. G., Lehrbuch der physiologischen Chemie, Leipsic, 2nd edition, 1850, ii, 367.
14. Albanese, M., *Arch. exp. Path. u. Pharmakol.*, 1895, xxv, 449.
15. Bodzynski, S., and Gottlieb, R., *Arch. exp. Path. u. Pharmakol.*, 1895, xxvi, 45; 1896, xxxvii, 385; *Ber. chem. Ges.*, 1895, xxviii, 1113.
16. Krüger, M., and Schmidt, P., *Ber. chem. Ges.*, 1899, xxxii, 2677.
17. Krüger, M., and Schmidt, P., *Z. physiol. Chem.*, 1905, xxxvi, 1.
18. Krüger, M., and Salomon, G., *Z. physiol. Chem.*, 1898, xxiv, 364; 1898–99, xxvi, 350.
19. Krüger, M., and Schmidt, J., *Z. physiol. Chem.*, 1901, xxxii, 104.
20. Leven, *Arch. physiol. norm. et path.*, 1868, l, 179.
21. Taylor, A., *Am. J. Med. Sc.*, 1899, cxviii, 141.

22. Burian, R., and Schur, H., *Arch. ges. Physiol.*, 1900, lxxx, 241.
23. Hess, N., and Schmoll, E., *Arch. exp. Path. u. Pharmakol.*, 1896, xxxvii, 243.
24. Haig, A., Uric acid as a factor in causation of diseases, London, 1896.
25. Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.
26. Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, 1912–13, xiii, 363.
27. Folin, O., and Denis, W., *J. Biol. Chem.*, 1913, xiii, 469; 1913, xiv, 95.
28. Clark, G. W., and de Lorimier, A. A., *Am. J. Physiol.*, 1926, lxxvii, 491.
29. Krüger, M., and Schmidt, J., *Z. physiol. Chem.*, 1905, xlv, 1.
30. Folin, O., *Z. physiol. Chem.*, 1904, xli, 223.
31. Lewis, H. B., and Nicolet, B. H., *J. Biol. Chem.*, 1913, xvi, 369.
32. Benedict, S. R., and Franke, E., *J. Biol. Chem.*, 1922, lii, 387.
33. Benedict, S. R., and Saiki, T., *J. Biol. Chem.*, 1909–10, vii, 27.
34. Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 633.
35. Funk, C., and Macallum, A. B., *Biochem. J.*, 1913, vii, 356.
36. Hill, H., *Ber. chem. Ges.*, 1876, ix, 370.
37. Hill, H., and Mabery, C. F., *Am. Chem. J.*, 1880, ii, 305.
38. Fischer, E., *Ber. chem. Ges.*, 1884, xvii, 328, 1776; 1895, xxviii, 2480; 1897, xxx, 559; 1898, xxxi, 3266.
39. Biltz, H., and Damm, P., *Ber. chem. Ges.*, 1913, xlvi, 3665; *Ann. Chem.*, 1914, ccccvi, 32; 1917, cccexiii, 141. Biltz, H., and Herrmann, L., *Ber. chem. Ges.*, 1921, liv, 1676. Biltz, H., and Heyn, M., *Ann. Chem.*, 1917, cccexiii, 179; 1921, cccexxiii, 185. Biltz, H., Marwitsky, K., and Heyn, M., *Ann. Chem.*, 1921, cccexxiii, 122. Biltz, H., and Strufe, K., *Ann. Chem.*, 1917, cccexiii, 124, 155.
40. Means, J. H., Aub, J. C., and Du Bois, E. F., *Arch. Int. Med.*, 1917, xix, 832.
41. Myers, V. C., Practical chemical analysis of blood, St. Louis, 2nd edition, 1924, 56.
42. Mendel, L. B., and Lyman, J. F., *J. Biol. Chem.*, 1910, viii, 115.

THE ESTIMATION OF UREA AND AMINO ACID NITROGEN IN ANIMAL TISSUES.*

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In the course of some work on protein metabolism it became advisable to estimate urea in animal tissues. Although we were drawn somewhat to the urease method we felt compelled for two reasons to employ other technique. In the first place, it was quite apparent that owing to the relatively large amount of ammonia in the tissue samples, the urea estimations would have to be accompanied by determinations of the preformed ammonia. Here we found ourselves presented by several additional difficulties, not the least of which was considerable uncertainty about the source of the tissue ammonia and the rate of its formation. From the work of Gad-Andresen (1) and of Hoagland and colleagues (2, 3) and by inference from the known lability of the blood ammonia precursors (4–10) we considered it likely that the preformed ammonia of the tissues would not be estimated easily. There was also the possibility that an unknown fraction of the tissue ammonia might arise, post mortem, from urea, leading obviously to a decrease in the latter. The observations of Gad-Andresen (1) on ammonia formation in muscle, although unconfirmed in some quarters (2, 3), justified the entertainment of such a fear. Finally, the recent work of Addis (11) throws grave doubt upon the permissibility of the urease method in the presence of arginase.

Of the remaining analytical methods, we regarded that of Fosse (12) as the most hopeful; although for simplicity in technique we wished to employ the same deproteinizing agent in the estimation

* The material in this paper is drawn from the thesis presented by Veon Carter Kiech as a candidate for the degree of Master of Arts at Stanford University.

of both urea and amino acid. We determined, therefore, to investigate the use of trichloroacetic acid for precipitation of the tissue proteins in place of Tanret's reagent which Fosse employed successfully in the analysis of serum. We hoped in this manner to extend the trichloroacetic method, previously described by one of us (13) for the estimation of amino acid nitrogen in tissues, to the determination of urea as well. The results were unsatisfactory, and demonstrated quite clearly that some other protein precipitant would have to be employed. We turned, therefore, to tungstic acid partly with the hope that minor modifications here and there might permit the whole Folin-Wu system of analysis to be applied to tissue extracts. Hammett (14) had already employed this precipitant preliminary to the estimation of creatine and creatinine in muscle extracts, while Denis (15) used it in determining the amino nitrogen content of fish muscle. In this method, we have been especially careful to avoid tissue autolysis, to shorten the time required for analysis, and to eliminate in the urea estimations even a moderate warming of the tissue and its filtrate.

Method.

The method now to be described was developed for use in the analysis of the whole carcass. We presume, however, that it is directly applicable to single tissues. We have used it for the estimation of amino acid nitrogen and urea in the liver, muscle, fetus, and entire body of the adult rat.

The animal is stunned by a sharp blow on the head and is at once put through a coarse meat chopper which has been previously cooled with liquid air. The mincings are chilled promptly by further treatment with liquid air, well mixed, and again run through the chopper. The coarse cutting plate is then replaced by a fine one, through which the frozen tissue is forced three or four times. Liquid air is added frequently. By this means the carcass may be reduced to a fairly uniform mass. The skin, bones, and hair cause no trouble.

6 to 10 gm. of the mixture, roughly measured, are then transferred to a cold iron mortar and pulverized to a fine powder. Occasional applications of liquid air are necessary to keep the material well chilled and brittle. Of the frozen substance 4 to 6 gm. are weighed out to the nearest cg. For this purpose we have

found a weighing bottle 40 cm. × 60 cm. to be most convenient, a subsequent transfer of the contents thus being avoided. Although the object to be weighed is very cold, we find that the error so caused need not exceed 1 or 2 mg. It is negligible when weighing to the nearest cg.

42 cc. of ice water, or if less than 6 gm. of tissue be employed enough water to give a final total volume of 60 cc., 6 cc. of ice-cold 10 per cent sodium tungstate, and 6 cc. of ice-cold sulfuric acid (0.66 N) are now added in the order named. The weighing bottle is vigorously shaken between additions. 2 to 4 minutes after adding the sulfuric acid, the contents are filtered. About 45 cc. of filtrate are obtained within 30 minutes.

Estimation of Urea.

20 cc. of the water-clear filtrate[1] are transferred to a 50 cc. centrifuge tube and treated with an equal volume of glacial acetic acid. The contents are stirred thoroughly. Finally, and with continued stirring, 2 cc. of a 10 per cent solution of xanthydrol in methyl alcohol are added. Within a minute or so, the body of the fluid becomes filled with a fine glistening precipitate of di-xanthydryl urea. After 1 hour, in which time, according to Fosse, precipitation of the urea is complete, the suspension is centrifuged and the supernatant fluid is decanted through a porous-bottomed filtration crucible,[2] attached to a suction flask and pump. The precipitate is then mixed with 10 cc. of 95 per cent alcohol, centrifuged, and separated from the supernatant fluid by decanting the latter into the filtration crucible. The washed precipitate is now transferred quantitatively with 6 to 8 cc. of alcohol to the filter. Filtration which hitherto has been quite rapid is now apt to be slow. It is therefore advisable to add the alcohol in several small portions and to permit the crucible to drain between additions. After the last of the alcohol has drained through, the suction is

[1] If the animal has not been fasted prior to killing, the tissue filtrate is very liable to be opalescent. This is probably due to the presence of glycogen. It does not seem to interfere in the subsequent estimations of amino nitrogen and urea.

[2] After trying several kinds of crucibles, we found the porcelain ones of Royal Berlin ware with set-in porous bottoms to be most suitable. These are distributed by Kreuger and Toll. Crucibles A2, size 00 (8 cc.) or 0 (15 cc.), are of convenient porosity and size.

continued until the dixanthydryl urea is fairly dry, as indicated by the whitening of the crystalline mat. 10 or 15 minutes later the crucible is transferred to an oven and heated for 30 minutes at 105°. It is then cooled in a desiccator over sulfuric acid. The entire precipitate may then be removed as a sheet from the bottom of the crucible and weighed directly upon the balance pan. If the crucible is not drained by suction until it is almost dry, the precipitate is very apt to adhere to the bottom of the crucible. In all instances, except with muscle filtrates, the precipitates have been crystalline, and white, and left no ash on incineration. In the case of muscle, the precipitates have been invariably yellowish and impure. This anomalous behavior of muscle is now under investigation. Whatever the cause of the difficulty may be, we have never experienced it with liver, fetus, or the whole adult animal.

The crucible, protected by a porcelain cup, is ignited for a few minutes and on cooling is again ready for use.

Estimation of Amino Acids.

20 cc. of the filtrate are transferred to a 100 cc. beaker and evaporated on a hot plate to about 10 cc. Two or three glass beads are needed to prevent bumping. 4 or 5 drops of 10 per cent sodium hydroxide are now added to the fluid and the boiling continued. By this means preformed ammonia is removed. After 2 minutes, an excess of glacial acetic acid (0.5 to 1.0 cc.) is added and the evaporation continued until the volume is reduced to about 2 cc. The concentrate is transferred quantitatively with 2 or 3 cc. of wash water to the Van Slyke amino nitrogen apparatus.

Although we have not observed any quantitative agreement between the two methods, it is sometimes more convenient to employ Folin's colorimetric method (16) for amino nitrogen. For this purpose, 1 cc. of the tungstic acid filtrate is diluted with 4 cc. of water and treated as the equivalent of 5 cc. of blood filtrate in Folin's method.

EXPERIMENTAL.

Use of Tungstic Acid as Protein Precipitant.

The conclusion drawn from many preliminary experiments in which trichloroacetic acid was employed as the protein precipitant

was that even moderate heating of strongly acid solutions of urea in very low concentration caused relatively great loss. We were therefore driven to try some other protein precipitant which would give a filtrate of low acidity, and which would require, at no time, the application of heat. Since it was observed that 20 cc. of a solution of tungstic acid (2 cc. of 10 per cent sodium tungstate, 2 cc. of 0.66 N sulfuric acid, 16 cc. of water) when treated with 20 cc. of glacial acetic acid and 2 cc. of 10 per cent xanthydrol remained perfectly clear throughout 1 day, it appeared that no trouble would be encountered through the precipitation of a xanthydrol-tungstic acid compound if tungstic acid were used for protein removal.

The first of the experiments which followed were directed to the determination of very small amounts of urea, 1 mg. or so. If the precipitate could be obtained in a form which might be transferred quantitatively to the pan of the balance, direct weighing would be possible and a relatively small quantity of tissue filtrate might be employed. By this means we hoped to eliminate the tedious and undesirable feature of concentrating the urea solution.

(*a*) To this end we attempted first the technique of Fosse, in which the author, by employing parchment paper as a filter, was able to separate the entire precipitate as a single sheet. Perhaps because we were unable to secure the proper kind of parchment or parchment paper, we never succeeded in its use. We resorted then to drying the precipitate in centrifuge tubes or on watch-glasses but found that it stuck most tenaciously. Other preparations of dixanthydryl urea were filtered through discs of hardened filter paper in Gooch crucibles, washed, and dried. Again no separation of the precipitate from the paper was possible. Finally we tried transferring the precipitate with a few cc. of alcohol to a small area of a porous plate. The precipitate dried rapidly and could easily be separated in the desired form. This suggested to us the use of crucibles with smooth porous bottoms and glazed sides. Observing the precautions mentioned earlier, we found that these crucibles served the purpose excellently. The precipitate could be loosened by tapping or by running a needle around the edge and then removed as a thin white disc to the balance pan.

(*b*) We determined next the optimum proportions of tissue suspension, sodium tungstate, and sulfuric acid.

4 gm. portions of tissue were treated with varying quantities

of sodium tungstate and sulfuric acid and enough water to give total volumes of 60 cc. The urea was precipitated from 20 cc. portions of the filtrates. The results are presented in Table I.

We concluded from this series that 2 cc. portions of the precipitants for 4 gm. of tissue were too small. The filtrate was cloudy and gave a heavy coagulum when boiled. Although 4 cc. portions

TABLE I.

Estimation of Urea in Tungstic Acid Filtrates.

10 per cent sodium tungstate.	Sulfuric acid (0.66 N).	Water.	Filtrate.	Urea per 100 gm. tissue.	Amino N per 100 gm. tissue.
cc.	*cc.*	*cc.*		*mg.*	*mg.*
2	2	52	Cloudy.		
4	4	48	Clear.	82.6	51.3
4	4	48	"	82.6	55.5
6	6	44	"	90.0	50.8
6	6	44	"	88.0	
8	8	40	"	92.3	52.5

TABLE II.

Estimation of Urea in Animal Tissues. Recovery of Added Urea.

Weight of sample.	Urea added.	Dixanthydryl urea.	
		Recovered from 20 cc. filtrate.	Calculated.
gm.	*mg.*	*mg.*	*mg.*
4.000	0	4.2	4.2
4.000	3.78	12.3	12.34
4.000	3.0	11.3	11.2
2.73	3.0	10.0	9.86

of the precipitants gave clear filtrates and appeared to be adequate, we felt it desirable to employ 6 cc. portions in the later experiments.

(*c*) We examined next the recovery of urea added in known quantities to tissue samples. The results are presented in Table II.

(*d*) The recovery of urea from tissue samples of varying size was examined next. 6 cc. portions of tungstate and sulfuric acid were employed. Water was added to make the total volume of suspension 60 cc. The results are given in Table III. We are in-

clined to regard the apparent increases in urea and amino nitrogen which are to be observed with increase of sample size as partly due to concentration effects. There is less water presumably in unit volume of filtrate from an 8 gm. sample than would be contained in the same volume of filtrate from a 4 gm. sample.

(*e*) We examined again the effect of variations in the quantity of urea by increasing the size of the tissue sample and by adding increasing quantities of urea to samples of equal size. These

TABLE III.

Effect of Varying Size of Tissue Sample.

Weight of sample.	Dixanthydryl urea.	Urea per 100 gm. tissue.	Amino N per 100 gm. tissue.
gm.	*mg.*	*mg.*	*mg.*
4.0	4.6*		40.2
4.0	6.3	67.5	43.3
6.0	9.4	67.2	43.1
6.0	9.4	67.2	45.2
8.0	14.0	75.0	46.4

*A portion of this precipitate adhered to the crucible.

TABLE IV.

Sample No.	Sample weight.	Tungstate and sulfuric acid. Amount of each.	Added urea.	Dixanthydryl urea.	
				Recovered.	Calculated.
	gm.	*cc.*	*mg.*	*mg.*	*mg.*
1	4.0	6.0	0	4.7	4.4
2	4.0	6.0	2.0	9.2	9.2
3	4.0	6.0	4.0	14.8	13.7
4	8.0	6.0	0	8.8	8.8
5	8.0	10.0	0	13.8	8.8

results are summarized in Table IV. With Sample 5 which was designed to serve a different purpose, the quantities of tungstate and sulfuric acid were greatly increased. The urea precipitate exceeded in weight the calculated value and on incineration was found to contain much inorganic matter. The other precipitates on incineration burned away rapidly leaving no trace of ash. This trouble is never encountered in the routine procedure which calls for 6 cc. portions of tungstate and sulfuric acid to 4 or 6 gm. sam-

ples of tissue. The dixanthydryl urea precipitate of Sample 1 stuck to the bottom of the crucible from which it could not be detached. The value 4.7, was determined by difference in the weight of the crucible before and after incineration. The corresponding calculated value (4.4) was determined from Sample 4.

(*f*) To 4 gm. portions of frozen tissue increasing and known quantities of totally hydrolyzed caseinogen were added. The

TABLE V.

Recovery of Amino Acid Nitrogen.

Sample weight.	Added amino N.	Urea per 100 gm. tissue.	Amino N per sample.	
			Found.	Calculated.
gm.	*mg.*	*mg.*	*mg.*	*mg.*
4.0	0	68.6	1.76	
4.0	0	68.6	1.63	
4.0	1.068	77.0	2.85	2.77
4.0	1.068	88.8	2.85	2.77
4.0	2.136	82.5	3.85	3.84
4.0	2.136	83.5	3.80	3.84

TABLE VI.

Tissue Autolysis.

Time of thawing.	Mg. per 100 gm. tissue.	
	Amino N.	Urea.
hrs.		
0	44.9	45.0
0.5	50.2	47.0
2.0	70.6	48.5
4.0	94.9	47.0

results of the analyses are presented in Table V. We are unable to explain the progressive increases in the urea values, for as is to be inferred from Table VI, an increase in amino acid concentration has little, if any, influence on the apparent urea content.

Rapid Autolysis of Tissue.

6 gm. samples of the frozen, powdered, carcass of an adult rat were weighed out and permitted to stand at room temperature for

varying times before adding the reagents. The results which follow in Table VI demonstrate that proteolysis proceeds with great rapidity. The constancy in the urea values during tissue autolysis confirms the findings of Hoagland and Mansfield (3) and does not support Gad-Andresen's theory of rapid ureolysis.

This experiment also demonstrates the need of prompt preparation of the protein-free filtrates in tissue analysis.

SUMMARY.

A method is described for the estimation of amino nitrogen and urea in animal tissues. The latter is determined gravimetrically as dixanthydryl urea by direct weighing. Tungstic acid is employed as a protein precipitant.

Several points of technique in the early portion of the work were contributed by Mr. Benjamin Simon, to whom acknowledgment is made.

BIBLIOGRAPHY.

1. Gad-Andresen, K. L., *J. Biol. Chem.*, 1919, xxxix, 267.
2. Hoagland, R., McBryde, C.N., and Powick, W. C., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 433*, 1917.
3. Hoagland, R., and Mansfield, C. M., *J. Biol. Chem.*, 1917, xxxi, 487.
4. Salomon, W., *Virchows Arch. path. Anat.*, 1884, xcvii, 149.
5. Biedl, A., and Winterberg, H., *Arch. ges. Physiol.*, 1902, lxxxviii, 140.
6. Folin, O., *Z. physiol. Chem.*, 1902–03, xxxvii, 161.
7. Medwedew, A., *Z. physiol. Chem.*, 1911, lxxii, 410.
8. Parnas, J. K., and Heller, J., *Biochem. Z.*, 1924, cliii, 1.
9. Luck, J. M., and Seth, T. N., *Biochem. J.*, 1925, xix, 357.
10. Parnas, J. K., *Biochem. Z.*, 1925, clv, 247.
11. Addis, T., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxv, 365.
12. Fosse, M. R., *Ann. chim.*, 1916, vi, 13.
13. Luck, J. M., *J. Biol. Chem.*, 1928, lxxvii, 1.
14. Hammett, F. S., *J. Biol. Chem.*, 1921, xlviii, 127.
15. Denis, W., *J. Biol. Chem.*, 1922, liv, 693.
16. Folin, O., *J. Biol. Chem.*, 1922, li, 377.

XUM

THE EFFECT OF BORATE ON THE OXIDATION OF GLUCOSE AND OTHER SUGARS.

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INTRODUCTION.

The experiments recorded in this paper were suggested by a consideration of the details of some experiments on carbohydrate metabolism. In order to minimize the liability of the bladder to infection following catheterization, it is common practice to finish the catheterization by irrigation with boric acid solution. The known behavior of boric acid in the presence of polyhydroxy compounds as well as the possibility of pH changes in the reagent used for sugar analysis led us to test the effect of boric acid on the determination of urinary sugar. We have found that the presence of boric acid causes errors of a magnitude which cannot be ignored.

A dog was catheterized and then injected subcutaneously with 1 gm. of phlorhizin dissolved in Na_2CO_3. The catheterization was repeated at intervals of 2 hours, the bladder being emptied each time as completely as possible but without washing. Sugar was determined in these urines by the Folin and Peck (1919) titration before and after the addition of the amount of saturated boric acid solution which would have been used in washing (25 cc.). The results are shown in Table I.

We have found no mention of the interference of borates with sugar determinations in the handbooks or in the literature of phlorhizin diabetes. While the majority of investigators apparently have not used boric acid irrigation, it has been used by some. It is desirable therefore to call attention to the possibility of errors

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in the determination of urinary sugar (see Table I) where proper precautions are not taken to prevent any but a small amount of the boric acid from entering the sample.

These observations on the interference of boric acid with the oxidation of sugar excited our interest to such an extent that we have studied the problem from several angles, but this paper reports in detail only the effects of boric acid on copper and iodometric methods of determining glucose and the common mono- and disaccharides.

Theoretical.

Since the addition of certain organic hydroxy compounds causes an apparent increase in the degree of dissociation of boric acid in

TABLE I.

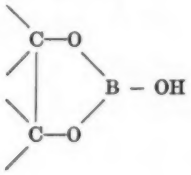
Urinary Sugar. Error Caused by Saturated Boric Acid.

Urine No.	Volume.	Glucose found.		Error.
		As taken.	With 25 cc. saturated HBO_2.	
	cc.	*gm.*	*gm.*	*per cent*
1	50	2.155	1.855	13.9
2	30	1.460	1.350	7.5
3	30	1.235	1.105	10.5

aqueous solution, it is possible after adding glycerol, mannite, invert sugar, or glucose in proper quantity to a boric acid solution, to titrate 1 hydrogen atom of the boric acid quantitatively. The apparently increased dissociation of the acid in the presence of polyhydroxy compounds is indicated by the position of the titration curves (Mellon and Morris, 1924), by the change in conductivity (Magnanini, 1890), and by the change in the reaction to phenolphthalein on adding these compounds to borax solutions (Biot, 1842). In some cases, *e.g.* mannite and sorbite, a change in the optical activity of the polyhydroxy compound may be observed (Fischer and Stahel, 1891).

The investigations of Magnanini have led to the belief that two hydroxyl groups on adjacent carbon atoms are necessary for the increase in conductivity of boric acid, and the work of Boeseken

(1913) has further indicated that these hydroxyl groups must be on the same side and in the same plane with respect to the carbon atoms for the most favorable effect. It was assumed that easily saponifiable esters were formed between hydroxy compounds and boric acid (Klein, 1878) and Van't Hoff (1908) gave the following ring structure to such esters:



Interaction of Glucose and Borates.

Boeseken (1913) believed that he had determined which of the two possible isomeric lactonic formulæ should be assigned to α-glucose and which to β-glucose on the following basis. α-Methylglucoside has only a slight negative effect on the conductivity of boric acid, which shows that the aldehydic hydroxyl group is necessary for the reaction with boric acid. While both α- and β-glucose increase the conductivity of boric acid, the α form causes the greater increase and this increase falls off at a rate equal to the rate of mutarotation. On the other hand the conductivity of the solution containing β-glucose increases with time at a rate equal to the rate of mutarotation. The structure with the hydroxyl group of the aldehydic carbon atom on the same side as that of the second carbon atom was therefore assigned to α-glucose. Irvine and Steele (1915) found however that 2, 3, 5, 6-tetramethylglucose (now regarded as 2, 3, 4, 6-tetramethylglucose (Charlton, Haworth, and Peat, 1926)) has practically the same effect on the conductivity of boric acid as glucose itself. The hydroxyl group on the second carbon atom therefore probably has no rôle in the formation of the borate ester, but according to these authors the second hydroxyl group necessary for the increase in conductivity is one formed on the addition of water to the lactonic oxygen atom.

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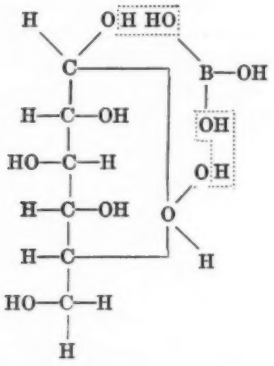
Thus:



This is in conformity with Armstrong's (1903) theory of the transformation of $\alpha \rightleftarrows \beta$-glucose through the oxonium derivative and Boeseken's opinion that the transformation occurs without rupture of the lactone ring. In any case, the formation of a borate ester of glucose involves the aldehydic hydroxyl group which is certainly of great importance in the oxidation of glucose.

The decrease in the optical activity of glucose on the addition of borax (Rimbach and Weber, 1905; Murchhauser, 1923) may be taken as additional evidence of ester formation, although the former authors ascribe part of the decrease to the effect of the alkaline reaction on the sugar. Boric acid itself has no apparent effect on the optical activity of glucose. If the alkali had an effect on the glucose in the sense of the transformation investigated by Lobry de Bruyn and Van Ekenstein, its neutralization should leave the optical activity unchanged, but if the low optical activity is a specific effect of borax, neutralization might restore the original optical activity of glucose. The data of Table II show the latter to be true, thus proving that under our experimental conditions none of the glucose was converted to mannose or fructose or suffered other irreversible changes in the presence of the alkaline borate.

The change of rotation with time in Solution 5 (Table II) is

very interesting in that it indicates the liberation of an excess of α-glucose. This phenomenon will be the subject of further work.

Conditions Affecting Formation of Glucose-Borate Compounds.

The equation representing the formation of an ester between glucose and boric acid may be written as follows:

$$C_6H_{12}O_6 + B(OH)_3 \rightleftarrows C_6H_{10}O_6{:}BOH + 2H_2O$$

That the amount of ester formed will depend on the concentrations of glucose and of boric acid is indicated by measurements of the conductivity at various concentrations of boric acid and of polyhydroxy compounds (Boeseken, 1913; Rimbach and Ley, 1922). The ester which is formed dissociates as a stronger acid than boric acid.

$$C_6H_{10}O_6{:}BOH \rightleftarrows C_6H_{10}O_6{:}BO^- + H^+$$

It may be stated as a general principle that reactions favored by the presence of alkali tend to produce acids which neutralize the alkali. Esters of alcohols and acids are hydrolyzed in the presence of alkali to give the free acid which neutralizes the alkali; lactones and acid anhydrides are converted by alkali to the acid forms and alkali brings about the formation of saccharic acids from the sugars. Applying this principle to the above reaction, the addition of alkali to a solution of boric acid and glucose should favor the formation of the ester of these two substances which is a stronger acid than boric acid. It was not surprising therefore to find that borax decreases the specific rotation of glucose but that boric acid has no measurable effect and therefore probably does not combine to an appreciable extent. (Rough calculations from the conductivity data of Boeseken show that probably not more than $\frac{1}{2000}$ of the glucose combines with boric acid, an amount certainly not detectable by the polariscope.)

Oxidation of Glucose.

In the oxidation of glucose by alkaline copper reagents, 5.5 to 6 equivalents of oxygen are utilized per mol of glucose. Numer-

ous products are formed by complex changes of the molecule; but one, gluconic acid, arises by direct oxidation of the aldehyde group of glucose. This acid may be oxidized further or the glucose may be split directly into fragments which are then oxidized. Blocking of the aldehydic hydroxyl group as in methylglucosides has a profound effect on the oxidation. Since boric acid or borates form esters through the aldehydic hydroxyl group, the prediction may be made that boric acid will interfere with the oxidation of glucose and this will be especially evident when the oxidation of glucose gives gluconic acid as the main end-product.

Since the oxidation of glucose by alkaline copper solutions is affected by the reaction of the solution (Visscher, 1926; Somogyi, 1926) and since boric acid as well as the ester of glucose and boric acid will alter the reaction of solutions to which they are added, the pH factor must be controlled before any specific effect may be ascribed to borates.

EXPERIMENTAL.

Oxidation of Glucose by Alkaline Copper Solutions.

Fehling's Solution.—In a series of experiments performed to determine the effect of the addition of boric acid on the oxidation of glucose by Fehling's solution, the Fehling-Soxhlet solutions were used in the manner described by Shaffer and Hartmann (1920–21) for the determination of glucose by the "cuprous" titration. The glucose used was the Difco, Anhydrous, C.P., or Pfanstiehl, C.P., Special Anhydrous. Boric acid was recrystallized three times from water and dried over $CaCl_2$. Merck's Blue Label boric acid as well as borax in equivalent amounts produced substantially the same effects. All determinations and blanks were run in duplicate. The results are shown in Table III.

In order to control the effect of neutralization of the alkali in the copper solution, we have determined glucose by Fehling's method after adding sulfuric acid. These results are shown in the last column of Table III. Since boric acid acts as if it were monovalent even in very alkaline solutions (Schmidt and Finger, 1908), we have assumed that the molecular and equivalent weights are the same and on this basis compared the effect of molar quantities of boric acid with 0.5 molar quantities of sulfuric acid. Since the

sulfuric acid is without effect under our conditions, it seems reasonable to believe that the interference of boric acid is not due to a mere change of reaction but is a specific effect of borate ions.

The presence of borate may interfere with the complete oxidation of glucose in the time of heating used, or the compound which

TABLE II.

Influence of Borate on Optical Acitivity of Glucose.

Solution No.		α	$[\alpha]$
		degrees	*degrees*
1	0.5 M glucose	9.59	53.3
2	0.5 " " + 0.5 M HBO_2	9.59	53.3
3	0.5 " " + 0.2 " " + 0.1 M NaOH	8.66	48.1
4	25 cc. Solution 3 + 25 cc. H_2O	4.24	47.1
5	25 " " 3 + 25 " 0.1 M HCl	4.80	53.3

α was determined at room temperature (25°), a 2 dm. tube and sodium lamp being used. The values given are the final values after mutarotation had ceased.

TABLE III.

Fehling's Solution. Influence of Boric Acid and Sulfuric Acid on Oxidation of Glucose.

Acid present.	HBO_2			H_2SO_4
	Glucose used.			
	0.5 mM	0.75 mM	1.0 mM	1.0 mM
m.-eq.	*mg.Cu*	*mg.Cu*	*mg.Cu*	*mg.Cu*
0.0	181	267.5	344	339
0.5	178		344	
1.0	176		342	
2.5	175	262.0	338	
5.0	173	257.0	332	
10.0	164	247.5	321	339
15.0	151	237.0	309	
20.0	149	225.7	295	338

it forms with glucose may be oxidized to give products which are different from those ordinarily formed. If the first postulate is true, increasing the time of heating should bring the amount of copper reduced closer to the amount reduced in the absence of borate. Table IV shows that increasing the time of boiling does not bring the copper reduced in the presence of borate closer to

that reduced when borate is not present and it appears, therefore, that the products of oxidation of the glucose in the presence of borate are different and less completely oxidized than in its absence.

The solutions (Table IV) were brought to boiling in 4 minutes and boiled for the lengths of time shown. Blanks and all determinations were run in duplicate under reflux condensers to prevent concentration of the solutions.

Micro Reagent of Shaffer and Hartmann.—Somogyi (1926) has shown that the optimal initial pH of the carbonate-bicarbonate buffer present in the micro reagent of Shaffer and Hartmann (1920–21) is 9.8 to 9.9. In the present work the original Shaffer-Hartmann reagent has been used because on the addition of boric acid the carbonate-bicarbonate buffer will approach the optimal condition rather than recede from it. The change in reaction of the buffer due to the addition of boric acid may be calculated from the dissociation constants of boric acid and the bicarbonate ion.

(1) $HBO_2 \rightleftarrows H^+ + BO_2^- \; K_{HBO_2} = \dfrac{[H^+]\,[BO_2^-]}{[HBO_2]} = 6.5 \times 10^{-10}$ (Clark, 1922).

(2) $HCO_3^- \rightleftarrows H^+ + CO_3^= \; K_{HCO_3^-} = \dfrac{[H^+]\,[CO_3^=]}{[HCO_3^-]} = 4.8 \times 10^{-11}$ " "

For the reaction:

$$CO_3^= + HBO_2 \rightleftarrows HCO_3^- + BO_2^-$$

(3) $$Km = \frac{[HCO_3^-]\,[BO_2^-]}{[CO_3^=]\,[HBO_2]} = \frac{K_{HBO_2}}{K_{HCO_3^-}} = 13.5$$

In the original reagent (5 cc.) there are 1.5 mM of Na_2CO_3 and 0.5 mM of $NaHCO_3$. Assuming complete dissociation for all salts, the concentrations (in mols per liter) will be, after the addition of 1 mM of HBO_2:

$$[HCO_3^-] = 0.1 + [BO_2^-]$$
$$[CO_3^=] = 0.3 - [BO_2^-]$$
$$[HBO_2] = 0.2 - [BO_2^-]$$

Substituting in equation (3) and solving:

$$[BO_2^-] = 0.19$$
$$[CO_3^=] = 0.11$$
$$[HCO_3^-] = 0.29$$
$$pH = 10.3 + \log \frac{0.11}{0.29} = 9.9$$

These calculations take no account of possible loss of CO_2 which, as Somogyi has shown, is a factor affecting the reaction of the mixture; nor do they allow for the dissociation as an acid of the small amount of glucose borate ester which might be present. This latter factor is negligible because if all the glucose (0.005 mM) combined with boric acid, the concentration of this stronger acid would be only 0.001 M in the undiluted reagent. The value of the pH given above is to be regarded only as an approximation which nevertheless is sufficiently exact for our purpose.

Judging by the pH of the buffer alone, the amount of copper reduced when 1.0 mM of boric acid is added should be greater than

TABLE IV.

Fehling's Solution. Influence of Time of Boiling.

Time of boiling.	1 mM glucose.	1 mM glucose + 10 mM HBO_2.	Difference.
min.	*mg. Cu*	*mg. Cu*	*mg. Cu*
2	334	316	18
5	335	317	18
10	340	322	18

TABLE V.

Shaffer-Hartmann Micro Method. Copper Reduced by 0.005 mM Glucose in Presence of Borate.

HBO_2 present, *mM*	0.00	0.025	0.050	0.250	0.500	1.000
Cu^{++} reduced, *mg.*	1.99	1.94	1.86	1.81	1.35	0.63

when no boric acid is present. Instead a marked decrease is found which we ascribe to the effect of the borate ion. Table V shows the number of mg. of copper reduced by 0.005 mM of glucose in 15 minutes in this solution when borate is present in varying amounts.

Experiments on the effect of time of heating on the amount of copper reduced showed that the reduction was not complete in 15 minutes when borate was present. Fig. 1 shows that the rate of oxidation as well as the amount of copper reduced are both decreased when borate is present.

The borate has two possible effects: (1) It reduces the concentration of free glucose which may be oxidized at a given time; or

(2) it forms a compound with glucose which may be oxidized directly to give end-products differing from those given by glucose alone and which therefore may reduce a different quantity of copper. The curves of Fig. 1 which show that borate decreases the amount of oxygen utilized by the glucose even though the

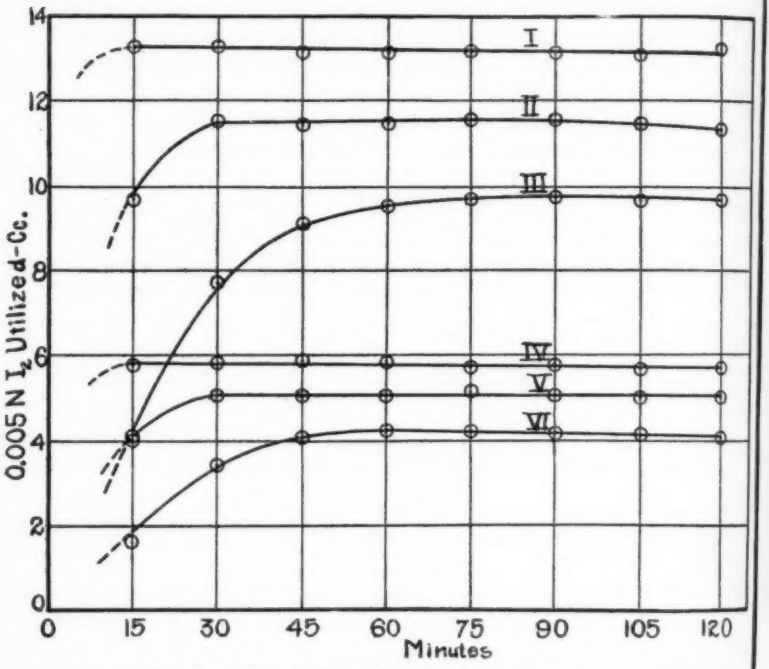


FIG. 1. The effect of boric acid on the reduction of copper in the Shaffer-Hartmann micro reagent. Curves I, II, and III were obtained with 0.01 mM of glucose alone, and in the presence of 0.5 mM HBO_2 and 1.0 mM HBO_2 respectively. Curves IV, V, and VI were obtained with 0.005 mM glucose, without addition, with 0.5 mM HBO_2, and with 1.0 mM HBO_2 respectively.

heating be continued for 2 hours seem to indicate that both effects are present, but that the second postulate is probably more important quantitatively.

Benedict's Qualitative Reagent.—If Benedict's qualitative reagent is used in the manner directed by Shaffer and Hartmann for

quantitative work, an interference with complete oxidation of glucose in the presence of boric acid is encountered.

0.25 mM of glucose and varying amounts of boric acid dissolved in 25 cc. of water were added to 25 cc. of the reagent. The solutions were brought to a boil in a covered flask in 4 minutes and boiling continued for 5 minutes. The results are shown in Table

TABLE VI.

Benedict's Qualitative Reagent. Influence of Boric Acid on Copper Reduced by 0.25 mM Glucose.

HBO_2, mM.	0.0	0.5	2.5	5.0	10.0	25.0
Copper reduced, mg.	80.3	78.5	73.2	63.4	40.2	25.0

TABLE VII.

Benedict's Qualitative Reagent. Influence of Borate on Rate of Reduction of Copper.

Time of boiling.	0.25 mM glucose.	0.25 mM glucose + 5.0 mM HBO_2.	0.25 mM glucose + 10.0 mM HBO_2.
min.	mg. Cu	mg. Cu	mg. Cu
5	80.3	63.4	40.2
10	79.6	66.3	51.5
15	80.3	67.2	56.7
20	80.1	67.5	58.0

TABLE VIII.

Folin Titration. Error Caused by Boric Acid Added to Glucose Solutions.

H_3BO_3	Titration.	Error.
per cent	cc.	per cent
0.0	2.53	
0.5	2.60	2.8
1.0	2.65	4.7
2.0	2.90	14.4

VI. By lengthening the time of boiling, more copper is reduced, but, as in the previous reagents, it does not reach the values found in the absence of borate. The solutions were brought to a boil in 4 minutes and boiling continued the length of time shown. Appropriate blanks were run. The results are given in Table VII.

Folin-Peck-McEllroy Method.—Since the Folin titration has been extensively used in this laboratory for the determination of sugar

in diabetic urines, we have determined the errors produced by the presence of boric acid in glucose solutions. Duplicate titrations usually checked within 0.02 cc. All solutions contained the same amount of glucose but variable quantities of boric acid. The results are given in Table VIII.

Oxidation of Glucose by an Acid Oxidizing Reagent.

Barfoed's Reagent.—Since it was shown in Table II that borax has a marked effect upon the optical activity of glucose but that boric acid produces no change in the specific rotation, it is interesting to compare the oxidation of glucose by an acid copper reagent in the presence and absence of boric acid. As shown in Table IX,

TABLE IX.

Barfoed's Reagent. Influence of Boric Acid on the Rate of Oxidation of Glucose.

The results are expressed in cc. of 0.02 N I_2.

Glucose, *m*M.....	0.025	0.025	0.05	0.05
HBO_2, *m*M.....	0	2.5	0	2.5
min.				
15	1.02	1.07	2.10	2.16
30	2.34	2.43	4.31	4.39
45	3.17	3.28	5.53	5.53
60	3.88	3.93	6.32	6.34
75	4.33	4.51	6.88	7.13
90	4.69	4.77	7.34	7.47

the presence of boric acid does not alter the rate of oxidation of glucose by Barfoed's reagent.

In passing, a few remarks on the technique of these determinations are inserted. Since some difficulties were encountered, the conditions under which satisfactory duplicates could be obtained with pure glucose solutions were worked out. The reagent used contained 0.16 mol of copper acetate and 0.16 mol of acetic acid per liter. 5 cc. of this reagent were heated with 5 cc. of the sugar solution and the copper reduced was determined by the "cuprous" titration method of Shaffer and Hartmann. Exactly 5 cc. of a solution containing 30 gm. of KI and 2.7 gm. of KIO_3 per liter (= 0.071 N I_2) were added to the copper solution containing the

Cu_2O followed by 2 cc. of saturated potassium oxalate solution and 1 cc. of 2.5 N H_2SO_4. After gently stirring the cuprous oxide precipitate with a glass rod to insure complete solution, the excess iodine was titrated with 0.02 N thiosulfate. The blank titration remained constant during the longest heating period but the amount of copper reduced appears to be altered by slight changes of the temperature of the water bath. The solutions containing the boric acid were heated at the same time as the controls containing no boric acid.

These data do not mean that combination between boric acid and glucose does not occur, but that due to rapid hydrolysis the small amount present does not affect the rate or extent of oxidation.

Oxidation of Glucose by Alkaline Iodine Solutions.

The oxidation of glucose to gluconic acid involves only the aldehyde group. When the aldehydic hydroxyl group of the lactone is blocked, as in glucosides, no oxidation occurs. If it is assumed that boric acid or borates react with glucose to form easily saponifiable esters involving the aldehydic hydroxyl group, the prediction may be made that boric acid will decrease the rate of oxidation of glucose to gluconic acid but will not interfere with the final completion of the reaction.

Romijn (1897) who first used alkaline iodine solutions to determine glucose advocated the use of a weak alkali to prevent the formation of iodate from the hypoiodite. Strangely enough he used borax for alkalinity and allowed 24 hours for complete oxidation. However, Willstätter and Schudel (1918) found that quantitative results were obtainable within 15 minutes if the borax was replaced by the gradual addition of an excess of 0.1 N NaOH. Cajori (1922) used sodium carbonate as the alkali and obtained quantitative results in 25 minutes.

Since the addition of boric acid to the carbonate solution will change the reaction of the solution, preliminary experiments were performed to ascertain the effect of the pH of the carbonate buffer on the rate of oxidation. Molar solutions of sodium carbonate and bicarbonate were prepared from the best grades of the salts available in the laboratory and the quantities of each measured and mixed in proper proportion to give several solutions contain-

ing 0.03 N Na^+ but of different pH. 125 cc. of exactly 0.1 N I_2 solution were measured into a 250 cc. volumetric flask and the glucose (450 mg. = 2.5 mM), which had been weighed and dissolved in a little water, was washed into the flask and the buffer solution added. This was taken as zero time. The flask was

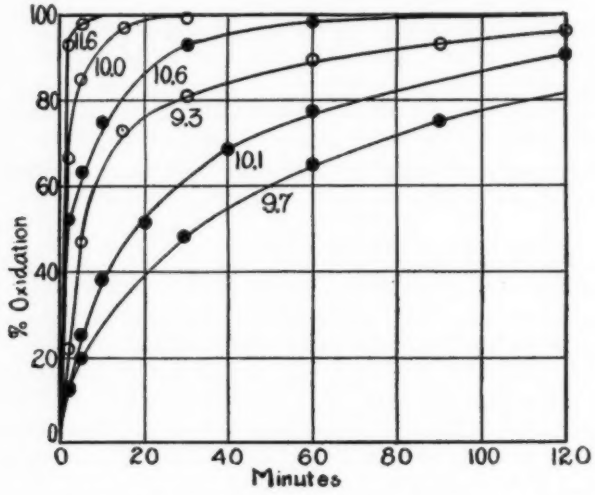


FIG. 2. The effect of boric acid on the rate of oxidation of glucose by alkaline iodine solutions. The figure below each curve represents the pH of the buffer present. The solid circles indicate the presence of borate. The exact compositions of the solutions are as follows:

Calculated pH.....	9.3	9.7	10.0	10.1	10.6	11.6
Na_2CO_3, M.....	0.025	0.15	0.075	0.15	0.15	0.15
$NaHCO_3$, M.....	0.250		0.150			
HBO_2, M.....		0.15		0.10	0.05	

rapidly filled to the mark, stoppered, and the contents mixed by inverting. Beginning at 2 minutes, 25 cc. samples of the solution were removed and immediately run into an excess of H_2SO_4; the iodine remaining in the sample was titrated with thiosulfate solution and the results expressed in per cent of glucose oxidized. Experiments were carried out at room temperature (22–25°).

The pH of the carbonate-bicarbonate buffer was estimated from the data of Auerbach and Pick cited in Clark (1922), but the actual pH of the solution during the oxidation differs from this because of the formation of anions from iodine. The results of this control series are shown with the results with carbonate-boric acid mixtures in Fig. 2. The experiments were performed in the manner described above and the pH of the buffer was estimated as previously described in the section on the micro reagent of Shaffer and Hartmann. Colorimetric tests showed that the pH values were in the calculated order and that the addition of glucose in the concentrations used had no appreciable effect on the pH.

The curves of Fig. 2 show the course of the reactions in carbonate-boric acid mixtures and carbonate-bicarbonate mixtures. Comparison shows that the rate is markedly decreased by the presence of borate. The curve for the carbonate-boric acid mixture of pH 10.1 shows a slower oxidation than the carbonate-bicarbonate of pH 9.3 in spite of the fact that increasing alkalinity favors the oxidation. Borate does not interfere with the completion of the oxidation but does interfere with its rate. The influence of temperature on the rate of oxidation of glucose by iodine has been tested by comparing the rates in carbonate-bicarbonate mixture of pH 10 and in carbonate-boric acid mixture of pH 10.1 in an ice bath, and in the incubator at 36°. The solutions were brought to the temperature of the bath before mixing. A rise in temperature increased the rate in both mixtures but the rate in the carbonate-boric acid mixture was always much slower than in the other buffer.

Influence of Borate on the Oxidation of Other Sugars.

The experimental data cited above would lead one to suspect that most other sugars would respond to the addition of boric acid by a decrease in reducing power toward Fehling's solution, and if they are aldo sugars by a decrease in the rate of oxidation by alkaline iodine solution. Table X shows that the oxidation of fructose, galactose, maltose, and lactose by Fehling's solution is less complete in the presence of borate than in its absence but the oxidation of arabinose seems to be unaltered.

The lactose, maltose, and galactose were the Pfanstiehl, C.P., Special brand, and the arabinose a C.P. Will Corporation sample

which gave the calculated values by Cajori iodine method. 0.5 mM of each of these sugars was used for the determinations given in Table X. Since the fructose was not C.P., the results with this sugar may not be entirely satisfactory.

The effect of borate on the rate of oxidation of various sugars by alkaline iodine solutions is shown in Table XI. The experiments were performed in the manner described previously with

TABLE X.

Influence of Borate on Copper Reduced in Fehling's Solution by Various Sugars.

Sugar, 0.5 mM.	No addition.	With 10 mM HBO_2.	Difference.
	mg.Cu	*mg.Cu*	*mg.Cu*
Glucose	177	167	−10
Fructose	314	305	− 9
Galactose	162	155	− 7
Arabinose	150	151	+ 1
Maltose	188	171	−17
Lactose	235	224	−11

TABLE XI.

Influence of Borate on Oxidation of Sugars by Alkaline Iodine Solutions.

Results are expressed in per cent of complete oxidation.

Sugar.	Glucose.		Fructose.		Galactose.		Arabinose.		Maltose.		Lactose.	
Buffer.	$CO_3^=$	BO_2^-	$CO_3^=$	BO_2^-	$CO_3^=$	BO_2^-	$CO_3^=$	BO_2^-	$CO_3^=$	BO_2^-	$CO_3^=$	BO_2^-
min.												
2	66.1	22.0	0.0	1.0	88.0	32.7	93.8	38.3	79.5	62.5	77.7	74.7
5	85.0	37.3	0.5	1.4	93.0	56.8	98.5	57.5	89.0	81.0	92.9	91.7
10	97.2	50.8			98.0	73.5	98.6	74.6	97.5	93.8	100.0	98.4
20	99.0	74.0	3.0	2.5	98.2	86.5	100.3	88.0	97.0	97.8	101.0	100.1
60	99.4	86.0	3.5	2.3	98.2	95.2	99.3	98.0	99.3	98.0		
120	99.9	94.5	6.3	2.3	99.0	98.2	101.5	99.3	99.3	100.0		

the carbonate-boric acid mixture (column headed $BO^=_2$) of pH 10.1 and the carbonate-bicarbonate mixture (column headed $CO^=_3$) of pH 10.0.

The rate of oxidation with hypoiodite of all sugars studied except lactose is definitely decreased; corresponding to this behavior of lactose it was noted by Klein (1878) that lactose does not change the reaction of borax solution to phenolphthalein.

The effect of borate on the oxidation of lactose by Fehling's solution may be accounted for by the effect of borate on the decomposition and partial oxidation products of lactose.

It has been claimed (Cajori, 1922) that pure fructose is not oxidized by alkaline iodine solution but our sample did utilize iodine. This may have been due to impurities or the slow oxidation of fructose itself or to the formation of aldo sugars through the effect of alkali. The oxidation of the latter has been shown to be depressed by borate.

The data of Table XI show that borate interferes to a greater extent with the oxidation of glucose than with the oxidation of galactose or arabinose in alkaline iodine solutions. The interferences in the latter sugars are practically equal and it is significant that each contains a favorably oriented pair of hydroxyl groups in the non-reducing part of the chain on the basis of the amylene oxide structure, whereas glucose does not contain similarly situated hydroxyl groups. Fischer (1895) found that α-methylgalactoside becomes optically active in borax solution whereas optical activity is not otherwise present (because of internal compensation (Hudson, 1909)). It is conceivable that the concentration of borate ion is reduced by ester formation involving the non-reducing groups of galactose and arabinose and as a consequence less of the ester involving the reducing group is present to interfere with its oxidation. We are aware that this argument requires substantiation at many points and that the extent of ester formation is not necessarily the same wherever the same group is involved but may depend also on the particular configuration of the remainder of the molecule. Thus borate has less effect on the oxidation of maltose which is 4- or 5-glucose-α-glucoside (Irvine and Black, 1926) than on glucose, although the group available for ester formation is the same in both cases. Borate has practically no effect on the oxidation of lactose and since lactose is 4- or 5-glucose-β-galactoside (Haworth and Leitch, 1918), two of the non-reducing hydroxyl groups of the galactose residue may have an effect analogous to the same groups in galactose itself and an interference similar in amount to that found with galactose might be expected. But, as in the case of maltose, disaccharide formation has a further unfavorable effect on the formation of an ester through the reducing hydroxyl group of the

glucose residue, and perhaps by virtue of these two unfavorable circumstances borate forms but a small amount of ester with the reducing hydroxyl group of lactose and consequently does not interfere with its oxidation. We hope that more extended study of the influence of borate on optical activity of the sugars may be correlated with the oxidation data and may help in its elucidation.

DISCUSSION.

Alkaline Copper Methods.—Borate, by forming a compound with the sugar being oxidized, hinders the oxidation of the aldehyde group by cupric ion. This probably changes the relative amounts of the various oxidation products which are formed and accounts for the decreased amount of copper reduced when borate is present. Some of the decomposition products which normally are oxidized further may form compounds with borate and be protected to some degree against this further oxidation. These decomposition products contain groups favorable to reaction with borate such as adjoining hydroxyl groups and α-hydroxy acids (Rimbach and Ley, 1922).

Alkaline Iodine Solutions.—The formation of borate esters interferes with the oxidation of the aldehyde group by decreasing the concentration of sugar available for oxidation by hypoiodite and therefore decreasing the rate of oxidation. As glucose is removed by oxidation, the ester is saponified by reversal of the reaction:

$$C_6H_{12}O_6 + B(OH)_3 \rightleftarrows C_6H_{10}O_6 : BOH + 2H_2O$$

making more glucose available for oxidation and allowing the oxidation to proceed to completion.

CONCLUSIONS.

1. The presence of borate decreases the amount of cuprous oxide formed by the oxidation of glucose, galactose, fructose, maltose, and lactose in Fehling's solution and decreases the rate and extent of oxidation in copper reagents which owe their alkalinity to sodium carbonate.

2. In an acid oxidizing reagent (Barfoed's) the presence of boric acid has no effect on the oxidation of glucose.

3. In the oxidation of glucose, galactose, arabinose, and maltose at the aldehyde group by alkaline iodine solutions, boric acid measurably decreases the rate of reaction. This does not appear to be the case with lactose.

4. The improper use of boric acid solution in collecting urines by catheter in the study of carbohydrate metabolism may give rise to serious errors.

BIBLIOGRAPHY.

Armstong, E. F., *J. Chem. Soc.*, 1903, lxxxiii, 1305.
Biot, *Compt. rend. Acad.*, 1842, xiv, 49, cited by Meyer.
Boeseken, J., *Ber. chem. Ges.*, 1913, xlvi, 2612.
Cajori, F. A., *J. Biol. Chem.*, 1922, liv, 617.
Charlton, W., Haworth, W. N., and Peat, S., *J. Chem. Soc.*, 1926, cxxix, 89.
Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.
Fischer, E., *Ber. chem. Ges.*, 1895, xxviii, 1155.
Fischer, E., and Stahel, R., *Ber. chem. Ges.*, 1891, xxiv, 2144.
Folin, O., and Peck, E. C., *J. Biol. Chem.*, 1919, xxxviii, 287.
Haworth, W. N., and Leitch, G. C., *J. Chem. Soc.*, 1918, cxiii, 188.
Hudson, C. S., *J. Am. Chem. Soc.*, 1909, xxxi, 66.
Irvine, J. C., and Steele, E. S., *J. Chem. Soc.*, 1915, cvii, 1230.
Irvine, J. C., and Black, I. M. A., *J. Chem. Soc.*, 1926, 862.
Klein, D., *Compt. rend. Acad.*, 1878, lxxxvii, 826, cited by Meyer.
Magnanini, G., *Z. physik. Chem.*, 1890, vi, 60.
Mellon, M. G., and Morris, V. N., *Ind. and Eng. Chem.*, 1924, xvi, 123.
Meyer, R. J., in Gmelin, L., Handbuch der anorganischen Chemie, Leipsic, 8th edition, 1926, pt. 13.
Murchhauser, H., *Biochem. Z.*, 1923, cxxxviii, 6.
Rimbach, E., and Weber, O., *Z. physik. Chem.*, 1905, li, 477.
Rimbach, E., and Ley, P., *Z. physik. Chem.*, 1922, c, 400.
Romijn, G., *Z. anal. Chem.*, 1897, xxxvi, 349.
Schmidt, C. L. A., and Finger, F., *J. Physic. Chem.*, 1908, xli, 412.
Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, xlv, 365.
Somogyi, M., *J. Biol. Chem.*, 1926, lxx, 599.
Van't Hoff, J. H., Lagerung der Atome in Raume, 1908, 3rd edition, 90, cited by Meyer.
Visscher, M. B., *J. Biol. Chem.*, 1926, lxix, 1.
Willstätter, R., and Schudel, C., *Ber. chem. Ges.*, 1918, li, 780.

XUM

A STUDY OF THE POSSIBLE RÔLE OF ALUMINUM COMPOUNDS IN ANIMAL AND PLANT PHYSIOLOGY.

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Plate 1.

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Aluminum is the most widely and abundantly distributed metallic element in the earth's crust, of which it is estimated to represent 7.3 per cent, whereas iron and calcium represent only 4.2 to 3.5 per cent respectively. Accordingly, aluminum must have been intimately associated with living matter throughout the ages. In recent years this element has assumed an increasingly important and conspicuous rôle in the human environment due to its use in the purification of water, in cooking utensils, in baking powders, and in medicinal products. Therefore, serious consideration has been given to its possible biological significance and to the effects of its salts in the diet.

Langworthy and Austen (1), Gonnermann (2), Bertrand (3), and others, have reported the presence of significant amounts of aluminum in a long list of plant and animal products. Osborne and Mendel (4) have reported better growth in white rats from diets containing "artificial protein-free milk" to which had previously been added traces of iodine, manganese, fluorine, and aluminum, than from the same "artificial protein-free milk" not containing these elements. Daniels and Hutton (5) have suggested as a result of experimental studies that aluminum may be essential to reproduction.

Such data as the above suggest that aluminum possesses definite physiological functions and that this element is an indispensable dietary constituent.

A different view is held by Gies (6) and his coworkers. They have since 1905 conducted repeated investigations of the dietary

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properties of aluminum compounds. As a result they seem convinced that aluminum compounds when present in the diet are absorbed out of the gastrointestinal tract and carried in the blood stream to different parts of the body with harmful effects.

In contrast to the findings of Gies and his coworkers are those of the Referee Board of Consulting Scientific Experts (7), of which Ira Remsen was chairman. These investigators studied the effects on human subjects of a dietary containing biscuits baked with the sodium aluminum sulfate type of baking powder. From these studies they concluded that aluminum residues present in biscuits baked with the sodium aluminum sulfate type of baking powder have no harmful effects on metabolism. Similar conclusions have more recently been expressed by Schmidt and

TABLE I.

Parts of Aluminum per Million.

Worker.	Date.	Egg white.	Wheat.	Corn.	Potatoes.
Theile, R.	1867	762.0			
Penney, M.D.	1879		6000–25000		
Myers, V. C., and Voegtlin, C.	1914		450	1700–1800	290
Gray, P. R.	1924	0.2–0.4	7.4–5.9	9.7–11.2	3.3
Sullivan, B., and Near, C.	1927		3.0		

Hoagland (8). These investigators found, furthermore, that all aluminum salts ingested were excreted in the feces.

The wide divergence of views and conclusions expressed by the above investigators on the biological and dietary significance of aluminum may be due in part to faulty methods of determining aluminum. It is interesting to note that in a chronological compilation such as is given in Table I there tends to be a progressive decrease in the amount of aluminum reported to be found in the different biological products indicated.

Since analytical chemistry has undergone improvement during the period covered by Table I, it may be assumed that the earlier published data are too high. In 1904 Langworthy and Austen published a compilation of data on the biological distribution of

aluminum. In the introduction of this publication these writers state:

> "In the older investigations, particularly those dealing with the mineral constituents of plants, data regarding aluminum are more abundant than in later works, and doubtless some of the aluminum reported came from impure reagents, from dirt contaminating the sample, or some similar cause. . . . The greater part of the material included in the compilation does not seem open to that objection, for, as time has progressed, analytical methods and chemical manipulation have improved, and there is no reason why determinations of aluminum made within recent years should not be fairly good."

However, it still seems to be a question whether there exist any trustworthy data on the biological distribution of aluminum. Further improvement in analytical methods or the selection of other and more applicable methods for determining aluminum in biological materials may demonstrate that even the most recent values in Table I are also too high.

Several difficulties may be anticipated in determining aluminum contents of biological materials by chemical methods. Undoubtedly the most serious of these difficulties is that of preventing contamination by aluminum from outside sources. Due to the wide-spread occurrence of aluminum, its complete absence from the best available chemical reagents and from the laboratory environment can hardly ever be achieved or assured even when the most rigid precautions are observed. Accordingly, it is always exceedingly difficult if not impossible for the analyst who uses chemical methods for determining aluminum in such small amounts as may be expected to occur in biological matter to produce data from which are precluded all possibilities of influence by aluminum from outside sources. In using chemical methods for determining aluminum in biological material there will therefore invariably be the probability that part or all of the aluminum reported was due to unavoidable contaminations. For ascertaining the presence or absence of small amounts and also for approximate estimations of such amounts of aluminum, the spectrographic method has the following advantages over all chemical methods: (1) sensitiveness; smaller amounts of aluminum can be detected by it; (2) specificity; the possibility of confusing other substances for aluminum is entirely eliminated; (3)

simplicity and rapidity of manipulations; (4) absence of all chemical reagents except atmospheric oxygen. Such conditions reduce to the lowest possible point all chances of aluminum contamination.

In view of these considerations the spectrographic method was used in this investigation. The details of the technique were as follows: The material was ashed in silica dishes supported on silica triangles over Bunsen flames. The spectrum of the resulting ash was excited in a 20,000 volt condensed spark between vertical copper electrodes by placing 20 to 30 mg. of the ash in a hollow of the lower electrode. The secondary circuit contained a self-induction coil which served to reduce the intensities of the lines due to air. The spectrum so produced was dispersed and recorded on plates by means of a Hilger E 1 quartz prism spectrograph. In the investigation reported in this paper this spectrographic technique was employed in ascertaining the biological distribution of aluminum and in conjunction with feeding tests for ascertaining the dietary properties of aluminum salts.

The *raies ultime* of aluminum are, according to de Gramont (9), the lines 3944.0 and 3961.5. These numbers are the names of the lines and their wave-lengths in Ångstrom units. The line 3961.5 is the first *raie ultime* because a smaller quantity of aluminum is required to produce it than any other line in the aluminum spectrum. This line persists after all the other aluminum lines have disappeared as a result of a progressive dilution of aluminum in the spectral source. The line 3944.0 is the second *raie ultime* because it is second to 3961.5 in the above respects.

It was obvious from the work of de Gramont that the purposes of the investigation could be served adequately and most conveniently by confining spectral examinations to the above two lines. A few preliminary spectrographic experiments of our own showed this to be so. Accordingly, only a portion of the spectrum sufficient to include these and a few adjoining lines has been reproduced in this paper.

In order to facilitate the identification of lines 3944.0 and 3961.5, and also in order to demonstrate the absence of these lines from the copper electrodes, all spectrograms were produced by means of a Hartman diaphragm. The operation of the Hartman diaphragm is illustrated by Fig. 1, which represents two typical

spectrograms. In each spectrogram the upper spectrum is that of the empty electrodes. The middle spectrum, or spectrum of the unknown, is that of the same electrodes with 20 to 30 mg. of the ash to be investigated in the lower electrode. The third or control spectrum is the same as or a continuation of the middle spectrum except that 1 drop of a 0.1 per cent solution of aluminum in form of the chloride was placed on the lower electrode previous to the third exposure and of course after photographing the middle spectrum. The two short and relatively heavy lines near the center and in the third or bottom spectrum are 3944.0 and 3961.5, the former being on the left and the latter on the right. The absence of these lines in the upper spectrum is evidence or proof that the electrodes do not contain aluminum. Their absence from the middle spectrum is evidence of the absence of aluminum in the ash, which was placed in the lower electrode.

In order to ascertain the sensitiveness of this spectrographic technique, or in order to ascertain the smallest quantities of aluminum which could be detected by it, under conditions of this investigation, the spectrograms in Figs. 2 and 3 were prepared. Each of the three spectrograms in Fig. 2 represents ash of 10 gm. portions of the same fresh whole egg preparation. Another and different whole egg preparation was used as a source of ash for the three spectrograms in Fig. 3.

The upper or first spectrogram in Fig. 2 is that of the ash of whole egg. In the middle spectrum of this spectrogram no trace of either aluminum line is present. Accordingly, so far as can be ascertained by this spectrographic technique, whole egg contains no aluminum. The second spectrogram in Fig. 2 is of the ash of 10 gm. of whole egg to which had been added previous to ashing, 1 ml. of a solution containing 0.001 per cent of metallic aluminum in form of the chloride. The egg previous to ashing, therefore, contained 1 part per million of aluminum. In the middle spectrum of this second spectrogram it can be observed that this concentration of aluminum (1 p.p.m.) was sufficient to produce both aluminum lines, although 3944.0 is rather faint. The third spectrogram in Fig. 3 is that of 10 gm. of whole egg to which had been added 2 ml. of the 0.001 per cent solution of aluminum, making a whole egg preparation containing 2 p.p.m. of metallic aluminum previous to ashing. In this spectrogram the aluminum

lines produced by the 2 p.p.m. are slightly but nevertheless distinctly heavier than those produced by 1 p.p.m. in the second spectrogram. The spectrograms in Fig. 2 show, therefore, that this spectrographic technique is easily capable of detecting 1 p.p.m. of aluminum in biological products. Furthermore, the differences between the second and third spectrograms in Fig. 2 suggest the possibility of approximating the quantity of aluminum present.

Fig. 3 contains spectrograms which show that this spectrographic technique is capable of detecting 0.5 p.p.m. of metallic aluminum. The first spectrogram in Fig. 3 is that of the ash of whole egg without addition of any aluminum. It represents, therefore, the same kind of ash as that represented by the first spectrogram in Fig. 2. In conformity with the first spectrogram of Fig. 2, the first spectrogram of Fig. 3 shows the absence of aluminum in whole egg.

The second spectrogram in Fig. 3 is that of the ash of 10 gm. of the same whole egg preparation used in preparing the first spectrogram. But to these 10 gm. was added previous to ashing 0.5 ml. of a solution containing 0.001 per cent of aluminum. This mixture represents, therefore, 10 gm. of whole egg containing 0.5 p.p.m. of added metallic aluminum. An examination of the second spectrogram in Fig. 3 will show that this concentration, *viz.* 0.5 p.p.m. of metallic aluminum, is sufficient to produce line 3961.6 but not 3944.0. The third spectrogram in Fig. 3 is that of whole egg ash containing 1 p.p.m. of aluminum added in the same manner. As is to be expected, this spectrogram contains both aluminum lines, as does the second spectrogram in Fig. 2.

For purposes of this investigation the following conclusions may be drawn from the experiments represented by the spectrograms in Figs. 2 and 3 concerning the sensitiveness of the spectrographic technique described above for demonstrating the presence or absence of aluminum in biological material.[1]

[1] In examining the spectrograms reproduced in this paper due allowance should be made for the fact that they represent the last reproducing step in a successive series of four. The original spectrum was first reproduced on a glass negative. The image on the negative was next reproduced on a paper print. The image on the paper print was then reproduced on a half-tone etching which finally reproduced the images on the accompanying plate. In every one of these reproducing steps the original spectrum loses some of

1. An aluminum concentration of less than 0.5 p.p.m. is demonstrated by the absence of both aluminum lines, since 0.5 p.p.m. is sufficient to produce line 3961.5.

2. An aluminum concentration of approximately 0.5 p.p.m. and less than 1 p.p.m. is demonstrated by the presence of line 3961.5 in the absence of line 3944.0.

3. The presence of both aluminum lines is evidence of an aluminum concentration of 1 p.p.m. or more.

Biological Distribution of Aluminum.

This spectrographic technique was applied to a number of plant and animal products in order to ascertain the biological distribution of aluminum. The following plant products were examined: wheat germ, yeast, navy beans, Lima beans, potatoes, carrots, and cottonseed meal. The animal products examined were hens' eggs and the following organs and tissues of the rat: liver, kidneys, spleen, testes, ovaries, bone, skeletal muscle, intestinal walls, skin, and lungs. All of these products, with the exception of rat skin, intestinal walls, and lungs, gave spectrograms identical with or at least equivalent to those illustrated by Fig. 1 in that neither aluminum line was present. Rat skin, intestinal walls, and lungs gave spectrograms which contained line 3961.5 but not line 3944.0. The spectrograms of these three products were equivalent to the second spectrogram in Fig. 3, which represents a concentration of 0.5 p.p.m. of aluminum.

This general survey indicates that aluminum is not a constituent of biological material. If present at all its concentration is less than 0.5 p.p.m. because this concentration is sufficient to produce line 3961.5, which was always absent except in the case of rat skin, intestinal walls, and lungs. In these three tissues the aluminum was apparently present but its concentration was less than 1 p.p.m. Otherwise line 3944.0 would have been present. The presence of such a trace of aluminum on the hair, intestinal wall, and in the lungs is to be expected. All of these tissues are always in intimate contact with dust, dirt, and other foreign material in which

its definition. The interpretations and conclusions expressed in this paper are based on examinations of the negatives which show delicate lines more distinctly than do the final half-tones.

aluminum is invariably present. Therefore a trace of aluminum in such tissues is more apt to represent adsorbed foreign matter than any normal tissue constituent.

Dietary Action of Aluminum Compounds.

Young rats were raised to mature ages on diets containing aluminum compounds. The rats so raised were compared with respect to growth, reproduction, and general well being with control rats raised under identical conditions on diets free from

TABLE II.

Composition of Diets.

	Control.	Aluminum chloride.	Baking powder.
Yeast	10.0	10.0	10.0
Casein	20.0	20.0	20.0
Salt Mixture 185*	4.0	4.0	4.0
Agar	2.0	2.0	2.0
Butter fat	8.0	8.0	8.0
Casein	56.0	55.4	53.0
Aluminum chloride		0.6	
Baking powder		0	3.0

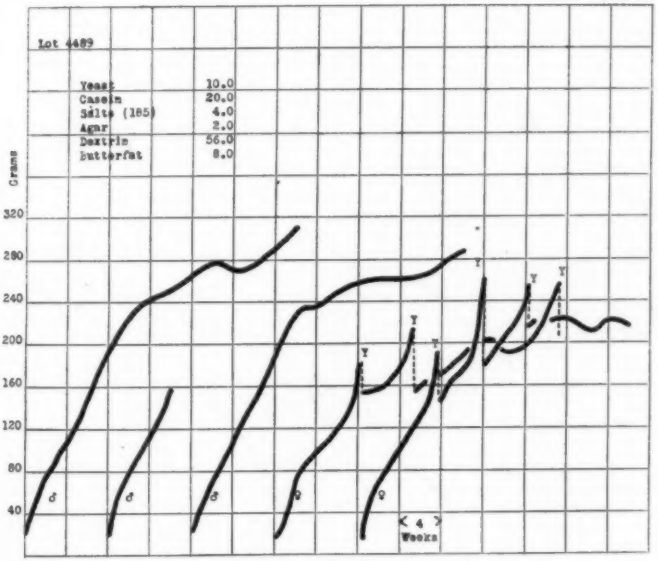
* Composition of Salt Mixture 185.

	gm.
NaCl	146.0
$MgSO_4$ (anhydrous)	225.0
$NaH_2PO_4+H_2O$	293.0
K_2HPO_4	805.0
$CaH_4(PO_4)_2+H_2O$	456.0
Fe citrate (ic)	100.0
Ca lactate	1098.5

aluminum but otherwise identical with the aluminum-containing diets. Two separate and independent experiments were carried out. In one of these the test diet contained 0.6 per cent of aluminum chloride ($Al_2Cl_6 12H_2O$) and in the other the test diet contained 3 per cent of a commercial brand of sodium aluminum sulfate, calcium acid phosphate baking powder, which had been artificially[2]

[2] This artificial decomposition was brought about by mixing with 30 gm. of the fresh powder, 15 ml. of distilled water and heating the resulting mixture at 100° for 30 to 45 minutes in a constant temperature oven.

decomposed or deteriorated previous to incorporation with the other dietary constituents. The composition of the control diet and the two test diets is given in Table II. The baking powder had previously been found by analysis to contain 2.1 per cent aluminum. Accordingly, 0.6 per cent of aluminum chloride and 3 per cent of baking powder represent respectively 0.067 and 0.063 per cent of aluminum in the metallic form. In feeding tests the



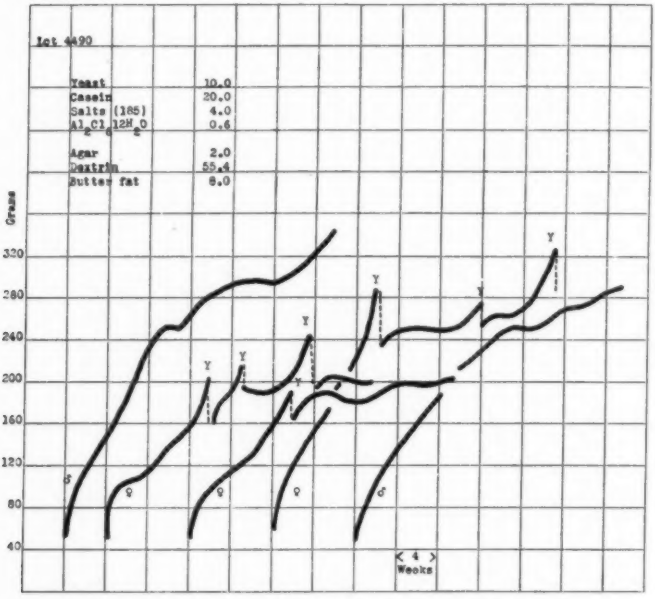
TEXT-FIG. 1. Growth records of control group of rats on aluminum-free diet.

difference between these two concentrations of aluminum may be disregarded.

The rats used in these experiments were born of our breeding stock and weighed 45 to 55 gm. each when started on the diets. Ten of these were placed on the control diet and thereby served as controls. Ten were placed on the aluminum chloride-containing diet, and six on the baking powder-containing diet.

The growth curves of these rats are given on Text-figs. 1, 2, and

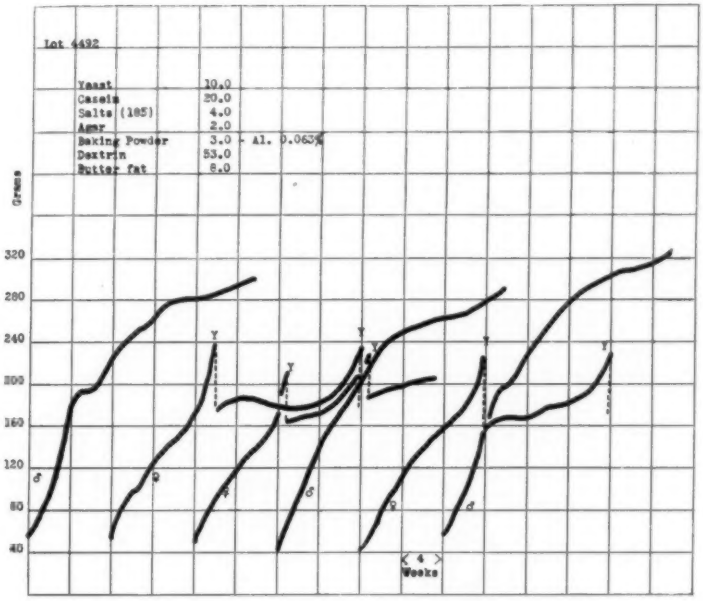
3. These curves also show frequencies of reproduction. Photographs of typical controls and tests are shown by Text-figs. 4 and 5. These charts and photographs show that rats receiving in their diets 600 p.p.m. of metallic aluminum either in form of the chloride or sodium aluminum sulfate, calcium acid phosphate baking powder are the same as the control rats with respect to growth, reproduction, and general appearance. The young of the



TEXT-FIG. 2. Growth records of group of rats fed aluminum chloride-containing diet.

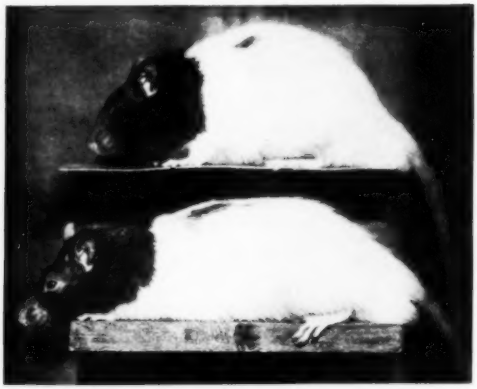
controls were in general discarded but a large number of the young of both of the test groups was examined for the presence of aluminum by the spectrographic technique already described. These examinations were applied to the ashes of the entire bodies of baby rats ranging in age from 1 to 7 days and to the ashes of individual organs of other second generation young after they had been weaned and had subsisted independently for 3 to 4 weeks on

their respective test diets. The ashes of the individual organs examined in this manner were those of liver, kidney, spleen, ovary, testis, skeletal muscle, bone, and intestinal wall. The ashes of the entire bodies of the young baby rats and the ashes of all individual organs except those of the skin, lungs, and intestinal walls gave spectrograms like those illustrated in Fig. 1, indicating the absence of aluminum down to less than 0.5 p.p.m. of the fresh

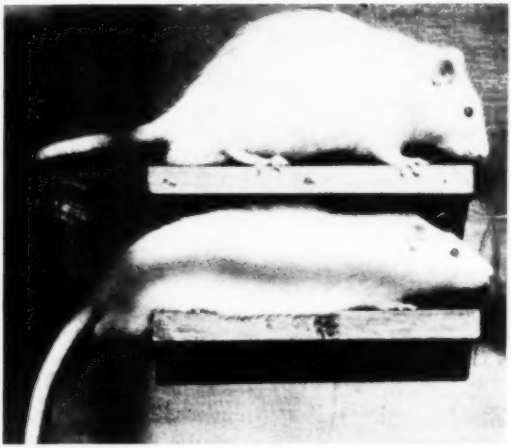


TEXT-FIG. 3. Growth records of group of rats fed aluminum in the form of sodium aluminum sulfate baking powder.

tissue. The spectrograms of the ash of skin contained line 3961.5, but not 3944.0, which indicates a concentration of 0.5 p.p.m. but less than 1 p.p.m. The spectrograms of the ash of intestinal walls contained both lines of such intensity as to indicate very little more than 1 p.p.m. It should be mentioned at this point that the intestinal walls previous to ashing had been slit open and gently agitated for 15 to 20 seconds in each of four successive 1



TEXT-FIG. 4. The rat at the top is the control rat; the lower rat received sodium aluminum sulfate, calcium acid phosphate baking powder.



TEXT-FIG. 5. The rat at the top is the control rat; the lower rat received aluminum chloride.

liter portions of distilled water so that extraneous matter was apparently absent.

The significance of aluminum in the skin, lungs, and intestinal walls has already been discussed. In order to ascertain the persistence with which aluminum in the diet adheres to or is absorbed by the intestinal wall, a series of spectrographic examinations was made of intestinal and stomach walls of rats raised on the three diets; *viz.*, the control diet, the aluminum chloride diet, and the baking powder diet. Typical spectrograms of these experiments are shown by Fig. 4. These spectrograms represent ashes of intestinal and stomach walls of second generation rats which had subsisted on their respective diets 3 to 4 weeks. Previous to ashing each of these alimentary tracts was washed in four successive 1 liter portions of distilled water in the manner already described. The first of these spectrograms is that of the ash of the intestinal tract of a second generation control rat about 2 months old. The second is that of the ash of the intestinal wall of a test rat of the same age which had subsisted on the control diet during the 3 days preceding the experiment. The third is that of the ashes of the intestinal tract of another test rat of the same age which had subsisted on the control diet during the 5 days that preceded the experiment. All spectrograms show the presence of aluminum though less than 1 p.p.m. As might be expected the second spectrogram shows the highest concentration. However, the third shows no more aluminum than does the first, which represents the aluminum content of the intestinal tract of a rat raised on a supposedly aluminum-free diet. Apparently, therefore, the intestinal tract does not absorb or combine permanently with aluminum in the diet, even though the diet contains large quantities, since aluminum which is swept out of the tract in as short a time as 5 days could only have been entrapped in a mechanical manner. In this respect no differences could be detected between aluminum in form of the chloride and in form of sodium aluminum sulfate baking powder residue.

As the experimental work was being terminated, the livers, kidneys, spleens, ovaries, and testes of four of the first generation test rats were examined for the presence of aluminum. At the time of these examinations, these rats had subsisted on the test diets for about 8 months. All of these organs were found to be

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aluminum-free or rather to contain less than 0.5 p.p.m. of the element, since their spectrograms were identical with those illustrated by Fig. 1.

During the progress of the feeding experiments, spectrograms were prepared of the ashes of the three diets and of the feces resulting from these diets. These spectrograms are shown by Figs. 5 and 6. It is interesting to note that the control diet appears free from aluminum but that the resulting feces show line 3961.5, indicating something less than 1 p.p.m. of aluminum. Apparently there was a very small trace of aluminum in the test diet, too small to be revealed by the spectrographic method but sufficient to represent 0.5 to 1 p.p.m. of the fecal concentrate.

The heavy aluminum lines of the ash of the aluminum chloride-containing diet are due to 0.1 per cent of metallic aluminum, as compared with 0.063 per cent of metallic aluminum in the baking powder diet. A concentration of 1 per cent of $Al_2Cl_6 12H_2O$ or 0.1 per cent metallic aluminum was used for the first 2 weeks of the feeding experiment. The concentration of the $Al_2Cl_6 12H_2O$ was then lowered to 0.6 per cent in order to equal more nearly the aluminum concentration in the baking powder-containing diet.

The spectrograms in Figs. 5 and 6 indicate, or at least suggest, that any aluminum in the diet is excreted without passing through the walls of the alimentary tract. The spectrograms representing the diet and the feces of the control rats indicate that any concentration of aluminum however small is never absorbed but always excreted.

DISCUSSION OF RESULTS.

As has already been remarked, previous studies of the biological and dietary properties of aluminum compounds have resulted in very diverse views and conclusions. These may be divided into three groups. According to one, aluminum compounds are very toxic and should therefore be excluded from the diet. According to a second group, aluminum is a normal constituent of a large number of both plant and animal tissues; the introduction into the intestinal tract of limited quantities of aluminum is therefore not only a normal and harmless occurrence but is probably even essential to life. According to the third group, to which belong the findings of the Referee Board of Consulting Scientific Experts,

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our normal consumption of foods, containing added aluminum, is not deleterious or injurious to health.

The present investigation has yielded results which indicate that the views of the first group are erroneous, and also that the second group is erroneous in its views regarding the wide-spread occurrence of aluminum in biological matter. However, the results of our investigation confirm the views of the Remsen board.

The following are the only conclusions indicated by the present study:

1. Aluminum is not a constituent of either plant or animal matter.

2. Aluminum compounds are not absorbed out of the stomach or intestinal tract when present in the diet.

3. Aluminum compounds when present in the alimentary tract do not form any union or compound with the stomach or intestinal walls.

4. Aluminum compounds in the diet in concentrations as high as 600 p.p.m. of the element aluminum exert no noticeably deleterious action on growth, reproduction, or general well being as judged by external appearance and autopsy.

These conclusions can probably not be regarded as final until after additional and confirming data have been obtained on a larger variety of materials and other animals. But until then there seems to be no other alternative than to accept them as tentative.

This investigation is one of a series on the dietary and other biological properties of supposedly biologically rare elements. A study of certain dietary properties of fluorine has already been reported from this laboratory (10). A consideration of the possible biological functions of manganese is now in progress and in the near future similar considerations will be given to zinc, boron, arsenic, and probably other elements.

BIBLIOGRAPHY.

1. Langworthy, C. F., and Austen, P. T., The occurrence of aluminum in vegetable products and natural waters, New York, 1904.
2. Gonnermann, M., *Biochem.* Z., 1918, lxxxviii, 401.
3. Bertrand, G., *Bull. Soc. hyg. aliment.*, 1920, viii, 49.

4. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.
5. Daniels, A. L., and Hutton, M. K., *J. Biol. Chem.*, 1925, lxiii, 143.
6. Gies, W. J., *Biochem. Bull.*, 1916, v, 151.
7. Referee Board of Consulting Scientific Experts of the United States, *U. S. Dept. Agric., Bull. 103*, 1914.
8. Schmidt, C. L. A., and Hoagland, D. R., *Univ. Calif. Pub. Path.*, 1919, ii, 215.
9. de Gramont, A., cited by Twyman, F., Wave length tables, London, 1923.
10. McCollum, E. V., Simmonds, N., Becker, J. E., and Bunting, R. W., *J. Biol. Chem.*, 1925, lxiii, 553.

EXPLANATION OF PLATE 1.

The two short relatively heavy lines at the center of the bottom spectra are the aluminum lines.

FIG. 1. Two typical spectrograms. Top spectrum, empty copper electrodes; middle spectrum, copper electrodes with ash to be tested on lower electrode; bottom spectrum, same as middle spectrum with 1 drop of 0.1 per cent solution of aluminum added.

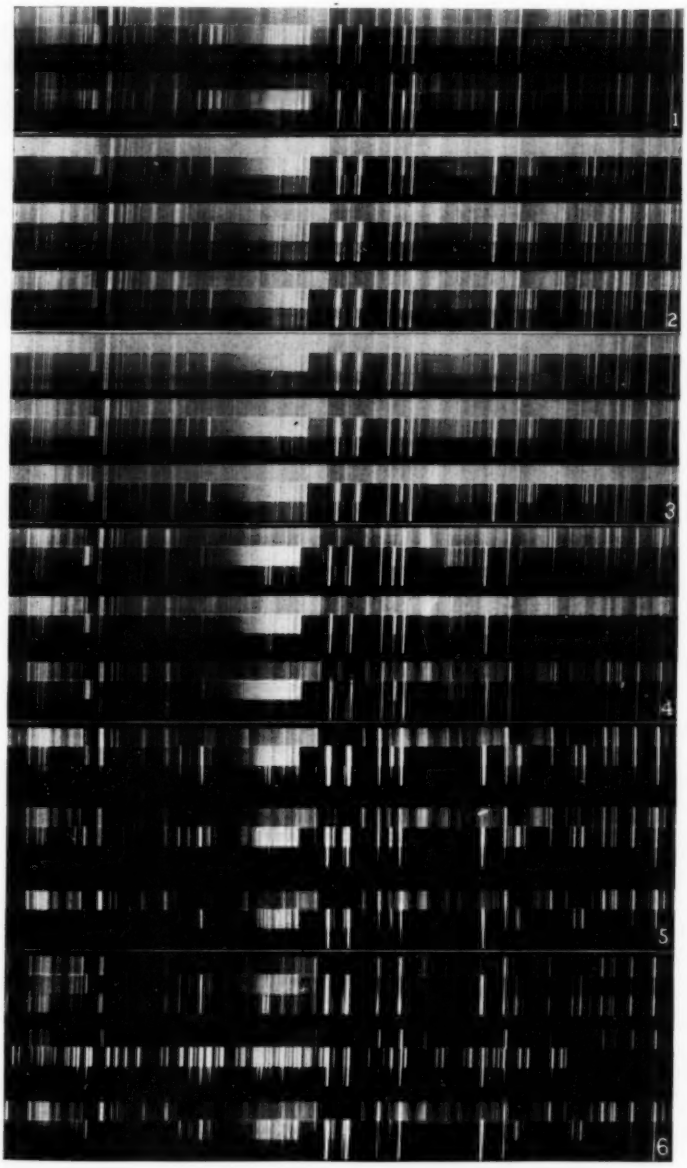
FIG. 2. Spectrogram standards. Top spectrogram, egg ash with no added aluminum; middle spectrogram, egg ash containing aluminum equal to 1 p.p.m. of the fresh egg; bottom spectrogram, egg ash containing aluminum equal to 2 p.p.m. of the fresh egg.

FIG. 3. Spectrogram standards. Top spectrogram, ash of whole egg with no aluminum added; middle spectrogram, ash of whole egg containing an amount of aluminum equal to 0.5 p.p.m. of the fresh egg; bottom spectrogram, ash of whole egg containing an amount of aluminum equal to 1 p.p.m. of the fresh egg.

FIG. 4. Spectrograms of ashes of alimentary tract. Top spectrogram, ash of alimentary tract of a control rat; middle spectrogram, ash of alimentary tract of a test rat which had subsisted on the control diet during the 3 days preceding excision of the tract; bottom spectrogram, ash of alimentary tract of a test rat which had subsisted on the control diet during the 5 days preceding excision of the tract.

FIG. 5. Spectrograms of ashes of diets. Top spectrogram, control diet; middle spectrogram, aluminum chloride-containing diet; bottom spectrogram, sodium aluminum sulfate, calcium acid phosphate baking powder-containing diet.

FIG. 6. Spectrograms of ashes of feces. Top spectrogram, feces of control rats; middle spectrogram, feces of aluminum chloride test rats; bottom spectrogram, feces of sodium aluminum sulfate, calcium acid phosphate baking powder test rats.



(McCollum, Rask, and Becker, Aluminum compounds.)

IRON IN NUTRITION.

V. THE AVAILABILITY OF THE RAT FOR STUDIES IN ANEMIA.*

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In order to extend the scope of investigations of iron metabolism in this laboratory we decided during the early summer of 1926 to attempt to use the rat as an experimental animal in addition to rabbits and chickens used heretofore. Early in 1925 we had done a limited amount of work using rats, but the attempts had not been carried to completion and we were in no position to make a comparison in the rapidity of production of anemia in our rats with those in other laboratories. Other workers had shown that anemia could not be produced rapidly in the rat. With white mice, Schmidt (1), feeding a low iron ration of rice and milk for three generations, secured a marked anemia in the second generation. In the third generation it was most pronounced. Happ (2), working in McCollum's laboratory, investigated the occurrence of anemia in rats fed deficient diets, among these diets being cow's milk and milk and bread. He stated that well balanced diets, deficient in iron, do not produce anemia in the rat in the first generation, "nor do diets consisting solely of cow's milk or milk and bread." He noted that slight anemia might occur in the second generation on these diets. Scott (3), before securing a well defined anemia in rats, had to breed them from dams which were fed white bread and milk from puberty till 10 months of age. The dams "showed practically no anemia themselves," but the young produced by them, when likewise fed bread and milk, developed a chronic anemia characterized by a low color index

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and by a tendency to spontaneous cure as the animals got older. Scott, before adopting this method, had tried (4) to produce anemia in rats by feeding them bread and milk from the time of weaning and by subjecting them to eight weekly bleedings, each amounting to 1 per cent of the body weight. Under these conditions the anemia which developed was quite transitory, persisting only for 2 to 3 weeks after the bleeding was stopped. Hence he abandoned this method as being too tedious and time-consuming and adopted the method already described.

In a personal communication from Dr. Mitchell at the Battle Creek Sanitarium, we learned that she too had not developed a pronounced anemia in rats during the first generation. The diet used by her, fully described later (5), was whole milk plus an addition of inorganic salts.

We believed, therefore, that we would have to secure our anemic young by breeding them from dams fed a low iron diet such as milk, and for this purpose we reserved during the course of about 2 months 120 young females. They were kept in groups of six or seven individuals in cages supplied with pine shavings litter. At the same time twenty-five young males were reserved to make breeding possible without disturbance or contamination of diet. All of these animals were started between 3 and 4 weeks of age, weighing generally from 50 to 60 gm.

The basal diet was whole cow's milk fed fresh every morning *ad libitum*. This milk was produced by a group of cows receiving wheat straw as their only roughage and a grain mixture composed of wheat and wheat gluten. Some of the cows of this group received a supplement of limestone to their grain ration and some received a small amount of cod liver oil. We used the milk from this group exclusively because we wished to have a constant supply from a known source. It may be added here, however, that we have also tried, in a few experiments, mixed milk from the large college dairy herd and have so far secured essentially the same results as reported below.

Since the animals were presumably to be a stock colony they were weighed only in 2 week periods. The cages were cleaned and fresh litter supplied at frequent intervals.

The rats grew well for a period of 4 to 6 weeks on the milk diet. At this time the weight generally became stationary and finally a

rapid decline set in. Coincident with cessation of growth it was noted that the animals were very pale and had what might be termed a bloodless appearance. This condition was particularly noticeable in the totally white rats and especially in their eyes and ears. Instead of the healthy pink color characteristic of the normal albino eyes, they showed a transparent paleness. The animals became quite inactive and huddled together in the cage. When they had been on the basal diet for 6 weeks fatalities began to occur in each group. In the first fifteen groups started, one or more deaths had occurred in each by the end of the 8th week. Of the 104 animals, representing these first fifteen groups, thirty-four had died by the end of the 8th week period on this basal diet.

TABLE .

Weights of Rats on an Exclusive Milk Diet, Recording the First Fatality in Each Group of Seven.

Group No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Rat No.	5	13	21	26	34	38	43	50	59	70	73	82	88	93	99
Wks. on milk diet.															
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	50	52	60	55	52	48	65	45	57	50	51	51	50	51	55
2	84	93	76	81	74	100	97	65	98	88	88	87	85	71	94
4	96	123	106	102	90	130	118	85	105	92	100	100	100	105	110
6	*	109	119	106	90	93	110	83	106	87	93	101	102	92	93
8		*	*	*	*	*	*	*	*	*	*	*	*	*	*

* Dead.

Other deaths followed in rapid succession and finally we discarded what few remained. Several other groups belonging to the colony we had set aside, which, however, had been started later in the summer, were taken in hand and placed on other diets before they had been on the basal ration so long and are not reported here.

In Table I is presented a summary of the weights and time of death of the first animal in each of the first fifteen groups.

When the animals began to exhibit pronounced symptoms as described and when some had died, we began to investigate the blood of the rats to determine whether they were anemic. These

investigations showed that indeed a distinct anemia had been produced. The red blood cell count of rats picked at random from the various groups ranged from 2,875,000 to 6,950,000 per c.mm., the majority of the counts being between 3 and 5 millions. The hemoglobin content of the blood was similarly reduced to between 5 and 9 gm. of hemoglobin per 100 cc. of blood. Both of these determinations are much below the normal values given by Donaldson (6) and Williamson and Ets (7) and also much below the figures determined on other rats in our laboratory, which were on a more varied and more complete diet.

TABLE II.

Hemoglobin and Red Blood Cell Count of Young Stock Rats Kept on Milk Diet.

Rat No.	Erythrocytes per c.mm.	Hemoglobin per 100 cc. blood.
	millions	*gm.*
10	4.17	
15	2.87	5.1
17	3.40	6.6
18	6.95	8.9
19	3.30	6.3
37	3.20	6.6
42	6.00	8.1
54	4.20	
101	4.85	8.7
102		8.9
103	4.75	7.4

In Table II are presented these first data on the blood condition of eleven rats picked from what survived of the first fifteen groups.

The erythrocytes were estimated in a Levy counting chamber with single Neubauer ruling, and the first hemoglobin determinations were made with the Fleischl-Miescher hemoglobinometer. Later we availed ourselves of the use of a Newcomer hemoglobinometer.

Having demonstrated that we could develop a pronounced nutritional anemia in our young rats in the course of 6 weeks to 2 months, we were, of course, glad to discard the original plan of breeding them from specially fed females. It has now become our regular procedure to wean the litters from our stock colony

at between 3 and 4 weeks of age (weight between 50 and 60 gm.) and to place them in cages provided with screen bottoms and feed them whole milk *ad libitum*. When the animals have been on this diet for 4 or 5 weeks they are segregated into individual cages and hemoglobin determinations are made weekly on different members of the litter. In this way the development of the anemia is followed. When the anemia has become very pronounced the animals are changed to some other experimental diet to determine corrective possibilities.

In Table III we have assembled the average results of the many hemoglobin determinations which we have made on young rats from our stock colony which received cow's whole milk as the sole

TABLE III.

Average Figures Showing Progressive Development of Anemia on Milk Diet.

	No. of individuals.	Hemoglobin per 100 cc. blood.
		gm.
At weaning	49	10.83 ± 0.24
After 3 wks. on milk diet	64	7.11 ± 0.10
" 4 " " " "	38	7.05 ± 0.13
" 5 " " " "	97	5.39 ± 0.09
" 6 " " " "	22	4.97 ± 0.25
" 7 " " " "	37	4.23 ± 0.15

diet. These figures were gathered over a considerable length of time on a large number of animals as we prepared them for experimental purposes. It will be noted that the anemia becomes more and more pronounced as time goes on, and if no change is made in the diet the animals die. Hemoglobin levels as low as 2 or 3 gm. per 100 cc. of blood may easily be secured. It must, however, be mentioned that the figures for hemoglobin at 6 and 7 weeks in Table III do not average as low as they should. This is due to the fact that many animals were changed from the milk diet to an experimental diet before the lapse of the usual 6 or 7 weeks time. Hence, *some* of the animals included in the *average* for 6 weeks and 7 weeks are those which had more hemoglobin-building reserves and required a longer period of time to develop the desired degree of anemia before the change to the experimental diet.

It may be noted here that we have in great measure dispensed with the erythrocyte count in the later work and have used only hemoglobin determinations. The former is a laborious and time-consuming operation and for our immediate purpose adds little or nothing to the information gained from an accurate hemoglobin determination. The operation of securing a blood sample is much simplified by the fact that only one pipette and one diluting fluid is used and it was mainly because of this greater expedition that we discarded the erythrocyte count. One person can perform the whole operation of taking a blood sample. The animal is first wrapped in a towel, the tail is snipped with a pair of sharp scissors, and the blood drawn by suction into the pipette. We have followed the suggestion made by Mitchell and Schmidt (5) of previously warming the tail of the rat in warm water and have found it helpful, especially in the case of very anemic individuals. After the sample has been taken, bleeding is stopped by momentarily applying a hot spatula to the wound.

DISCUSSION.

The fact that the young rats in our colony develop a pronounced nutritional anemia in such a relatively short time is doubtlessly due to a lessened storage or reserve of certain factors in their bodies. This in turn brings into discussion the handling and feeding of the females which make up the stock colony. The stock ration used by us has been described elsewhere (8) and need not be discussed here. Suffice it to say that the diet of our stock females is carefully controlled, as are the other conditions under which they live, and nothing in the way of table scraps or vegetable refuse is allowed them as is reported from many laboratories. It may be recalled, also, that the diet of our stock females includes considerable quantities of whole milk and it is possible that because of this the intake of the mixed ration is reduced. This may be a primary factor in determining the amount of hemoglobin-forming substance with which the young rats are supplied at birth.

The possibility of a difference in the nutritive properties of the milk used by us as compared to that used by other workers should also be kept in mind. So far as we have tried other samples of milk we have secured essentially the same results, although this is a point on which we are doing further work.¶

The females of our stock colony are not in any degree anemic themselves. We have never found any evidence of such a condition nor any reason to suspect it. The young rats themselves when weaned are not apparently anemic. It is true that the average figure for rats at this age as determined by us is lower than that of Williamson and Ets (7). The average found by them on 77 animals between the ages of 20 and 39 days was 12.62 ± 0.20 gm. Our figure is 10.83 ± 0.24. It remains to be seen whether this difference is significant. We are continuing to gather data on the hemoglobin level of our young rats at weaning.

SUMMARY.

Young rats, produced in our stock colony, when weaned at the age of 3 to 4 weeks and placed on a whole (cow's) milk diet develop a profound anemia in the course of 6 to 8 weeks. Their hemoglobin titer is reduced, in that time, from 10.83 gm. per 100 cc. of whole blood to 4 gm. or less. This stands in contrast with the reports of other workers who have found it necessary to carry rats into the second generation before anemia made its appearance.

BIBLIOGRAPHY.

1. Schmidt, M. B., *Verhandl. path. Ges.*, 1912, xv, 91.
2. Happ, W. M., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 163.
3. Scott, J. M. D., *Biochem. J.*, 1923, xvii, 166.
4. Scott, J. M. D., *Biochem. J.*, 1923, xvii, 157.
5. Mitchell, H. S., and Schmidt, L., *J. Biol. Chem.*, 1926, lxx, 471.
6. Donaldson, H. H., The rat, Philadelphia, 2nd edition, 1924.
7. Williamson, C. S., and Ets, H. N., *Am. J. Physiol.*, 1926, lxxvii, 480.
8. Steenbock, H., *Science*, 1923, lviii, 449.

IRON IN NUTRITION.

VI. IRON SALTS AND IRON-CONTAINING ASH EXTRACTS IN THE CORRECTION OF ANEMIA.*

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In the immediately preceding paper of this series we have described (1) the production of a profound nutritional anemia in young rats. To summarize briefly, it may be stated again, that young rats when weaned at 3 to 4 weeks of age (weighing 50 to 60 gm.) and placed on a diet of cow's whole milk develop, in the course of several weeks, a marked anemia characterized by very low hemoglobin levels. This anemia in our rats develops so rapidly that after 6 to 9 weeks of an exclusive milk diet the majority of the animals succumbs. The production of such an early anemia we believe to be a unique experience because other workers have emphasized the necessity of keeping animals on a milk diet to the second generation. In consequence of this distinctive reaction we were placed in a particularly fortunate position allowing us to unravel some of the sequence of factors operative in hematopoiesis. It will be recalled that previous work was carried out with rabbits and chickens. While these species can be used for this work as the results from this laboratory indicate, as a whole, they are not so well standardized nutritionally and genetically, which led to many experimental failures. These had to be balanced by numerous repetitions before unequivocal data were secured. Furthermore, with the rat we have experienced no

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difficulty in securing quantitative consumption of the various supplementary additions to the basal milk diet which gave us our base line of operations.

Preliminary Experiments.

In starting our studies of the factors operating in the cure and prevention of anemia, our first experiments were necessarily of a reconnoitering nature. Although this laboratory had already been at work on the problem of iron metabolism for some time (2, 3), we were here dealing with correction of anemia in a different species, and needs must go back to first principles.

In the anemia that we produce in our animals there is operating the factor of iron starvation. In view of this and also in view of the results of Mitchell and Schmidt (4) we naturally included in our first experiments a study of the effect of inorganic iron salts. We also studied the ash of dried lettuce since this is an iron-rich material which we had found peculiarly effective in the cure of anemia in rabbits (3). We furthermore included materials from an animal source, such as dried beef liver, dried beef kidney, and dried beef muscle, and yellow corn and wheat as plant materials which, if found corrective, undoubtedly provided a more complicated situation than the ashes or purified iron salts.

Preliminary Experiments on Inorganic Iron.

The iron salts used were ferric chloride (Mallinckrodt's ferric chloride lumps) and ferrous ammonium sulfate (Baker's C.P.). These two salts represented extremes of the average commercial products, one, the chloride, being a product not especially purified, while the other, the ferrous ammonium sulfate, was a most carefully purified preparation, being labelled by the manufacturer as 100 per cent pure. For making up solutions the ferric chloride was assumed to have the formula $FeCl_3 \cdot 6H_2O$. Both salts were fed in standard aqueous solutions. Usually 1 cc. was added to the first feeding of whole milk in the morning and no more milk was given till the animal had consumed this. Consumption was always good.

The two iron salts were first fed at a level of 2 mg. of Fe daily (6 days per week) to each animal. This amount we considered to be in excess of the animal's needs for iron. Abderhalden (5) in

1900 stated that he considered 0.4 mg. of Fe daily was sufficient to take care of the iron needs of a rat, and Mitchell and Schmidt (4) have had success in the cure of the anemia in their rats by using

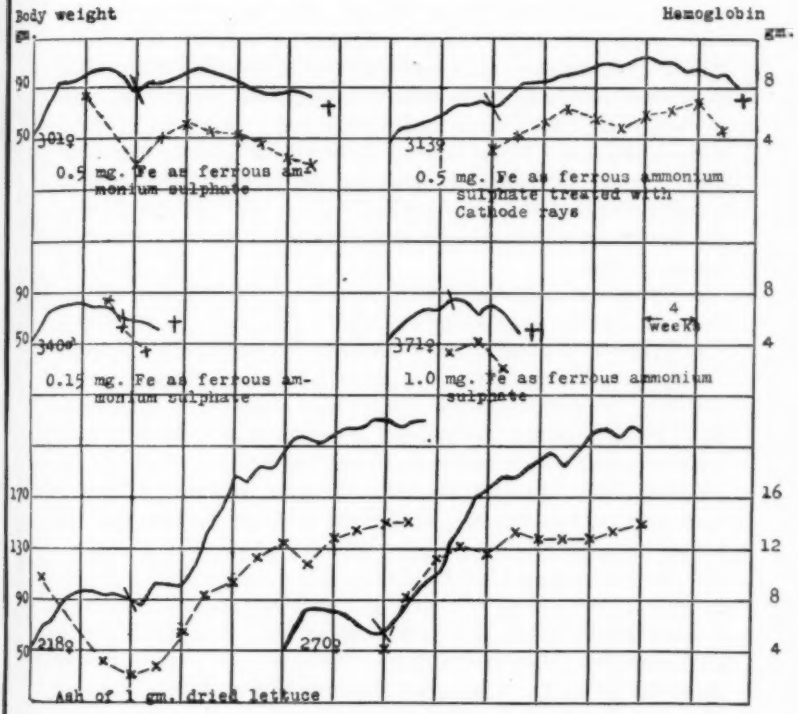

CHART I. Results of adding inorganic iron and ash of lettuce to basal diet of cow's whole milk. These additions were all made 6 days per week. In this and the following charts the solid line represents body weight, the broken line, hemoglobin values in gm. per 100 cc. of blood. The line across the curve for body weight indicates the point at which addition was made to the basal diet; the dagger at the end of the curve denotes death.

this amount. As we wished first of all to determine decisively the effect of inorganic iron, we chose this larger amount, realizing that much smaller amounts might be effective.

Later as more animals became available, we studied the effect of smaller amounts of inorganic iron added as ferrous ammonium sulfate to the basal diet of cow's whole milk. These levels were 0.1, 0.15, 0.5, and 1.0 mg. of Fe daily (6 days per week). The results secured showed that in no case were any of these smaller additions of iron effective in correcting the anemic condition of our animals. Seventeen animals which received these lower amounts all showed only slightly increased hemoglobin production and all died. In Chart I are recorded graphically the records of four of these animals. These four are chosen because of the fact that they survived for relatively long periods, which is particularly true in the case of Rats 301 and 313. These two animals showed the maximum effect secured from feeding these additions of inorganic iron.

It is to be noted that Rat 313 received a specially prepared solution of ferrous ammonium sulfate. This was used on the supposition that possibly the variability in results from different sources of iron was due to variation in amount of active *versus* inactive iron in the sense that these terms were used by Baudisch (6). We accordingly considered it worth while to subject iron salts to certain forces well known to affect atomic and molecular structure. To this end, therefore, a small amount of ferrous ammonium sulfate was subjected to the action of cathode rays, at a short distance from a Coolidge tube for a period of 1 minute. The salt was prepared for the exposure by dissolving 200 mg. of it in a small amount of water on a 2 inch watch-glass and then evaporating it to dryness over a water bath to obtain a thin layer spread over the watch-glass. The exposure to the rays was made for us by Dr. Farrington Daniels of the Department of Physical Chemistry of this institution. After exposure the salt was dissolved in water with the aid of a few drops of pure sulfuric acid and made up to volume. That it had not been activated is shown by the record of Rat 313 (Chart I) which is not essentially different from that of Rat 301 receiving the untreated salt.

Preliminary Experiments with Ash of Dried Lettuce.

Concurrently with the experiments on inorganic iron we also studied the effect of the ash of dried lettuce on hemoglobin building. The preparation of this ashed material has already been

described (3). Generally 100 gm. of dried lettuce were ashed at a time in an electric furnace, powdered, and stored in a desiccator.

The amounts necessary for individual feedings were weighed out daily and rubbed up with a small amount of milk as the first morning feeding. It was first introduced at such a level that 1 gm. of the dried lettuce was fed daily (6 days per week). Five animals were used in testing this material. The results were quite uniform and showed that the lettuce ash was very effective in correcting the anemia. In Chart I are recorded graphically the records of two animals, Rats 218 and 270. It may be noted there that the addition of the ash of 1 gm. of dried lettuce to the basal diet of cow's whole milk was sufficient to restore the hemoglobin titer to normal and to maintain it there, allowing the animal to grow to maturity.

The iron content of our dried lettuce was quite high and it was calculated that the addition of the ash from 1 gm. of it introduced about 1.4 mg. of Fe. This Fe intake was high as compared to the amount received by the rats on the previously fed levels of inorganic iron. Hence, later, a few experiments were started to determine whether smaller amounts of lettuce ash would suffice for the cure of this anemia as well. With levels of Fe intake of 0.1 and 0.15 mg. of Fe daily a few very anemic animals did not live long enough to give us definite results.

Preliminary Experiments with Beef Liver, Beef Kidney, Beef Muscle, Yellow Corn, and Wheat.

For the sake of brevity and convenience these five materials will be discussed together. They were chosen because they represent a wide variety in food substances. There was, of course, good reason to include the beef products because Robscheit-Robbins and Whipple (7) in studying the effect of various beef products on the regeneration of hemoglobin in dogs, found pronounced differences in the various organs and tissues, and because Minot and Murphy (8) had such success with beef liver feeding in the treatment of pernicious anemia in man. We have already shown its effectiveness in the cure of nutritional anemia in rabbits but it remained to be seen what effect it would have with the rat. The yellow corn and wheat were included because of the great importance of these two cereals in human and animal nutrition.

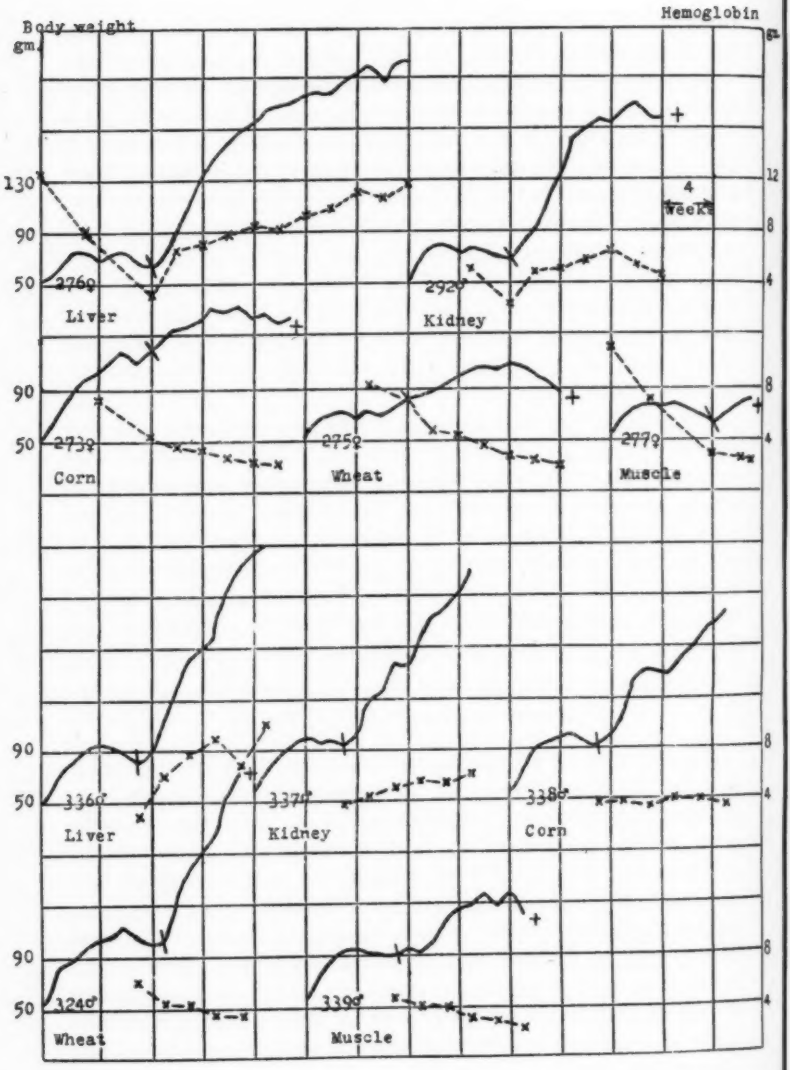

CHART II. A comparison of the efficiency in hemoglobin building of three beef products and two cereals. In the upper group of rats the five materials were fed at a level of 0.5 gm., 6 days per week. In the lower group the materials were fed at a level at which they each introduced 0.15 mg. of Fe, 6 days per week.

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Our idea was to choose a series of varied substances, determine their different values in the cure and prevention of the anemia with which we were working, and then attempt to explain these differences on the basis of their organic or inorganic make-up.

The beef liver, beef kidney, and beef muscle (beef round) were secured from a local meat market. They were sliced, dried in galvanized iron pans at about 70–80°, and then were finely ground and stored in stoppered glass jars. The yellow corn and wheat were also finely ground and similarly stored. The quantities for individual feeding were weighed out daily and added to the first feeding of milk in the morning. The consumption was always good.

All five materials were first fed at a level of 0.5 gm. per rat 6 days per week. In later experiments they were fed at different levels but with equal iron intake. Beef liver was taken as the basis, which showed by analysis that an intake of 0.5 gm. introduced about 0.15 mg. of Fe. The only substance among the five with a higher iron content than the liver was beef kidney. This necessitated feeding about 0.35 gm. of dried beef kidney, about 0.75 gm. of dried beef muscle, slightly less than 2 gm. of the wheat, and more than 2 gm. of the yellow corn per feeding. In testing each of these materials five, six, and even more animals were used.

The results of these experiments of which typical records are given in Chart II showed unmistakably the high value of beef liver in the correction of this nutritional anemia in rats. Not only did the animal respond with steadily increasing hemoglobin titer, but its physical well being and outward appearance were vastly improved and the growth curve went up very steeply. By comparison with beef liver, it can be noted, that beef kidney was less valuable, and that beef muscle was practically inert. The yellow corn and wheat caused increased growth, but apparently were unable to cause a similar increase in the hemoglobin content of the blood and in most cases the animals died.

It seemed quite remarkable to us that the low intake of 0.15 mg. of Fe which the feeding of 0.5 gm. of liver introduced (less than 1.0 mg. of Fe per rat per week of 6 days) was sufficient to maintain such pronounced growth and at the same time increase steadily the hemoglobin content of the blood. Increased growth of body tissues meant increased blood volume and it appeared,

therefore, that the animal was able to make very efficient use of the small amount of iron contained in the liver diet. It is not to be assumed that this level of iron intake was optimal since it was only after a period of 12 to 14 weeks that the hemoglobin level of the blood was what might be regarded as normal. The point was clear, however, that this iron level when fed as liver was much more effective than similar quantities of iron fed as kidney, muscle, corn, or wheat, and certainly much more effective than even much larger quantities of iron obtained from the inorganic iron salt, ferrous ammonium sulfate (see Chart I). Naturally the question then arose, was the efficacy of the liver in building hemoglobin due to its having the iron in a more available form or was it due to some accompanying substance or substances? If the latter, was the accompanying substance or substances organic or inorganic in nature? Since we had already found one inorganic material, namely the ash of dried lettuce, which served to cure this anemic condition in our animals, might it not be that the inorganic residue of liver would prove equally potent? It was with these questions in mind that we initiated further experiments.

Comparative Antianemic Effect of Iron in Different Combinations.

The possible variable rôle of iron in different combinations was called forcibly to our attention by the results of Mitchell and Vaughn (9). They observed a general difference between soluble and insoluble iron compounds but solubility evidently was not the only factor for even among the soluble compounds they observed obvious differences. The acetate, for instance, stood out pre-eminently as an efficacious source of iron. To rule out differences caused by possible contamination with hemoglobin-building impurities we were convinced that the only way to secure unequivocal data was to prepare various iron salts from a common source of iron, preferably of highest purity, by using reagents of highest purity. We accordingly prepared the chloride, sulfate, acetate, citrate, and phosphate in ferric form as described below.

Five portions of 2 gm. each of pure iron wire were weighed out and to each were added in a beaker 30 cc. of strong nitric acid (Mallinkrodt's reagent) and 15 cc. of distilled water. The nitric acid brought about very quick solution and oxidation to the ferric condition. Each of the five solutions was evaporated to dryness

on the sand bath and to each a small quantity of hydrochloric acid (Mallinkrodt's reagent) was added and again evaporated to near dryness. More hydrochloric acid was added and the solutions boiled to expel the last traces of nitric acid. At this stage, the solutions were diluted and ammonium hydroxide added to precipitate the iron as hydroxide. These mixtures were in turn boiled to expel the excess of ammonia, and finally the ferric hydroxide was filtered off and repeatedly washed. In this way five portions of ferric hydroxide were secured.

To each of these portions of ferric hydroxide was now added a calculated quantity of one of the following acids: hydrochloric (Mallinkrodt's reagent), sulfuric (Mallinkrodt's C.P.), acetic (Merck's C.P.), citric (Merck's C.P.), and phosphoric (Mallinkrodt's U.S.P.). During the slow addition of this calculated quantity to each of the portions of ferric hydroxide, the mixture was digested and stirred over a low flame. By adding a measured excess of acid, complete solution was obtained in the case of the chloride, sulfate, acetate, and citrate. With the ferric hydroxide-phosphoric acid mixture prolonged digestion succeeded in converting the hydroxide largely to insoluble white ferric phosphate, but even in the presence of a considerable excess of phosphoric acid the resultant precipitate maintained a reddish brown tinge suggesting the presence of a very small amount of ferric hydroxide. All five preparations were diluted to a volume such that 1 cc. contained 1 mg. of Fe. The ferric phosphate was fed as a suspension, being vigorously shaken each time before it was measured out.

The five salts were fed at a level of 0.5 mg. of Fe 6 days per week to a total of six litters of animals. Four of these litters had been on the basal diet of cow's whole milk for a period of slightly more than 5 weeks before the addition of the iron salts. At this time their average hemoglobin level was slightly more than 6 gm. per 100 cc. of blood. In the other two litters the addition of iron salts was made when they had been on the basal diet of milk only 3 weeks, at which time their average hemoglobin level was about 7.5 gm. per 100 cc. of blood. These hemoglobin values are by no means low when compared to the low levels that we can secure by leaving the animals for a longer time on milk. We chose to

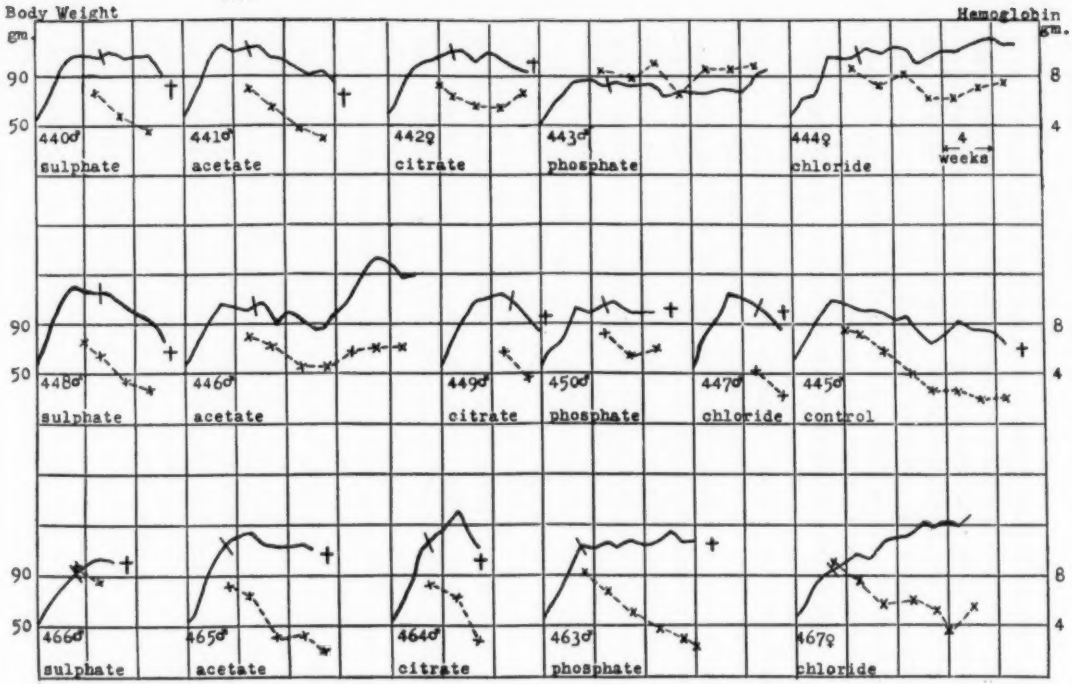

CHART III. In this chart are shown three of the six litters of rats that received as an addition to the basal diet 0.5 mg. of Fe 6 days per week from the five iron salts.

make these iron additions at a time when the hemoglobin-building reserves of the animals were still fairly high so that the inorganic iron might show a maximum effect.

In Chart III are presented graphically correlated hemoglobin and growth records of three of the six groups. These results are quite uniform and show that no one of these five inorganic salts of iron stands out from the others and that all completely fail to increase materially the hemoglobin of the blood. The insoluble ferric phosphate gave results in no way different from the more soluble salts. A majority of the animals died in from 4 to 8 weeks after the addition was made and those that survived for longer periods showed no significant improvement in either physical well being or hemoglobin titer.

Attention may be drawn to the record of Rat 443 in Chart III. This rat showed probably the best record of any in the whole series as far as hemoglobin is concerned. Its hemoglobin titer when the addition was made was 8.4 gm. per 100 cc. of blood. In opposition to the maintained level of hemoglobin, however, is the very poor growth response of the animal. Until the last week its body weight was always below 90 gm. and much of the time below 80 gm. This animal received the insoluble iron phosphate.

Attention may also be directed to the record of Rat 445. This was one of the control animals in the series which received no addition to the basal diet. It outlived all its litter mates except one, surviving much longer than usual for animals so fed. The interesting thing about its record is that during the last 6 weeks of its life its hemoglobin level was always below 3 gm. per 100 cc. of blood.

The results of the experiments which we have just described, added to those described in the preliminary experiments, and many others which we have made from time to time, lead us to state that, *using this level of iron intake alone*, we have never succeeded in materially correcting the nutritional anemia with which we are working. It must not be assumed from this that iron is not a factor in the cure of this condition. The point that we make is that, apparently, it is only one of several factors. As will be shown later, iron is a necessary element, but relatively small amounts will suffice in the presence of other substances.

CHART III. In this chart are shown three of the six litters of rats that received as an addition to the basal diet 0.5 mg. of Fe 6 days per week from the five iron salts.

Further Experiments on the Ashes of Certain Materials and on Acid Extracts of These Ashes.

While the experiments on the effect of the various iron salts on hemoglobin regeneration were still in progress, we studied in turn the inorganic residue from various food materials. It will be recalled that in our preliminary experiments we had found pronounced differences in the blood-regenerating powers of plant and animal tissues investigated. We had found dried beef liver to be very effective—like the ash of lettuce, but yellow corn, among others, was found to have a much smaller effect. However, as we have reported, the amount of iron intake in the experiments on the ash of lettuce was high as compared with that introduced with beef liver and corn, and the possible complicity of organic constituents in liver and corn made it impossible to compare these three materials directly. We, therefore, decided to compare them on the basis of their inorganic constituents alone and also on the basis of equal iron intake. This, we considered, would be the first step in determining whether the iron contained in one was in a more available form or whether the iron from one was more contaminated with other materials of an inorganic nature effective in hemoglobin building.

We also deemed it important to ascertain whether acid extracts of the ashes of these materials would have the same effect as the ashes themselves. If these extracts were as efficacious as the ashes from which they were made, it would settle the question of varied availability of the iron since the iron in all the extracts should be equally available when prepared with the same acid. Further, if the extracts were to be found equal in activity to the ashes of the three materials, it would serve as an important step in the concentration and isolation of the active material. Added to these considerations was the fact that the feeding and consumption of a solution is easier and more complete than an insoluble residue, particularly where milk is the basal diet.

Lettuce, upon ignition, leaves a porous ash which is easily scraped from the crucible and powdered. The ashing of corn and liver is much more difficult, particularly in the case of liver because of its high phosphorus content and fusible nature. To overcome this difficulty with liver we resorted to adding calcium

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carbonate to the dried liver before igniting. The amount added at first was 4 gm. to 100 gm. of dried liver. This mixture when ashed gave us a residue which we could handle with ease. We dispensed with the feeding of corn ash directly because, due to its low iron content, it required a relatively enormous amount for each feeding. We used only an acid extract of corn ash instead.

These extracts were prepared by adding a measured amount of strong hydrochloric acid to the ashed residue in each case, digesting for a sufficient length of time, diluting slightly, and filtering off the insoluble residue. The insoluble material was thoroughly washed and the resulting filtrate and washings diluted to such a volume that either 0.5 or 1 cc. contained 0.5 mg. of Fe. The various extracts were not always clear solutions, since on diluting some of them a slight precipitate settled out. In such cases the extract was always vigorously shaken before any quantity was measured out for feeding. For obvious reasons, the quantity of acid used was kept at a minimum. In this way we made four preparations; one from lettuce ash, one from the ash of liver ignited with calcium carbonate, one from the ash of liver ignited alone, and one from corn ash.

In these experiments seven materials were fed and compared, namely (1) ferric chloride, (2) ash of lettuce, (3) HCl extract of the ash of lettuce, (4) HCl extract of the ash of corn, (5) ash of liver ignited with calcium carbonate, (6) HCl extract of the ash of liver ignited with calcium carbonate, and (7) HCl extract of the ash of liver ignited alone. For the most part they were fed in such amounts as were required to furnish 0.5 mg. of Fe. The results in hemoglobin production are presented in Table I and graphically, correlated with weight relations, in Charts IV, V, and VI.

It seems to us unnecessary to discuss the data here presented at any great length. All the additions made in these experiments carried equal amounts of iron, and most of the iron was present in five of these additions in the same form—ferric chloride; but it is seen that the hemoglobin level of the animals receiving only ferric chloride gradually falls and that death in these animals eventually supervenes. Contrasted with this is the rapid increase in the hemoglobin of the animals receiving the iron from the other sources. Along with the hemoglobin increase goes increased

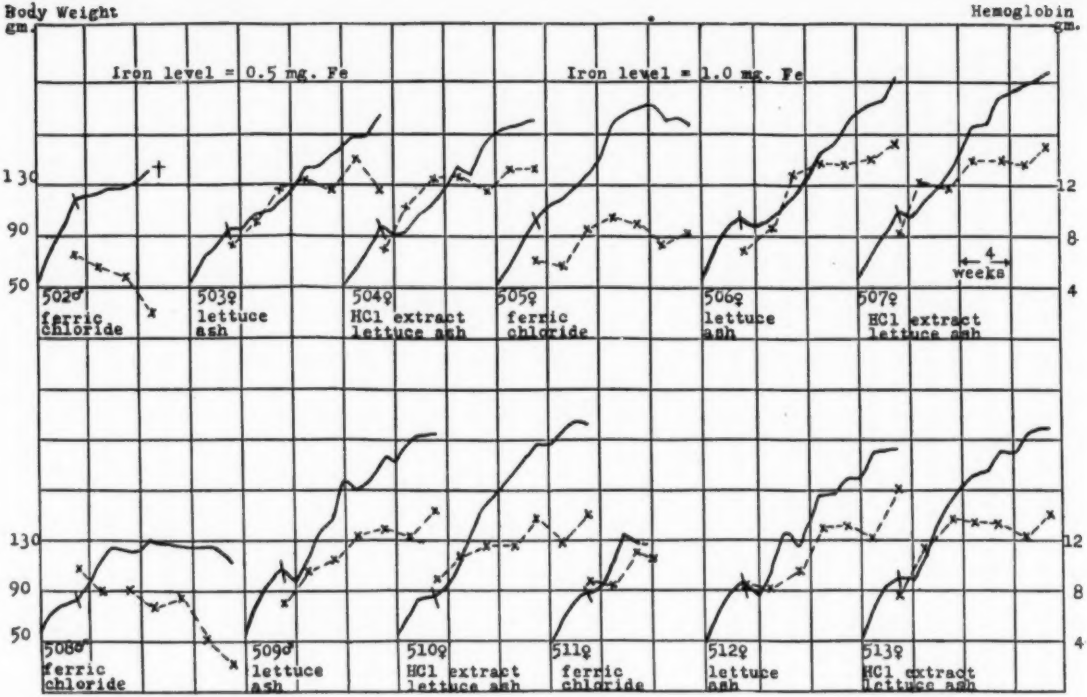

CHART IV. Comparison of hemoglobin-building value of iron from ferric chloride, lettuce ash, and the hydrochloric acid extract of lettuce ash, at two levels of iron intake. These additions were made to the basal diet of cow's whole milk 6 days per week. Rat 511 ♀ was killed at the point indicated because of a peculiar dry, scaly condition affecting the eyes, ears, and paws which we feared might be infectious.

bodily vigor and increased growth. This improved physical well being is a very noticeable feature following the addition of any potent material.

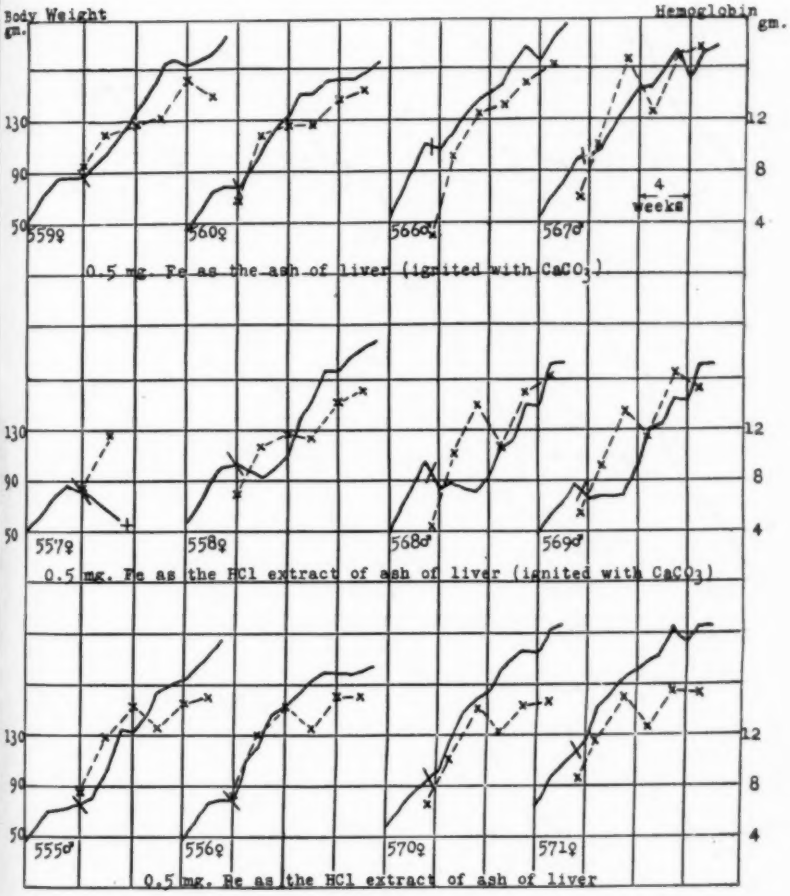

CHART V. This chart shows the very excellent results secured by adding to the basal diet of cow's whole milk iron contained in the ash of beef liver or hydrochloric acid extracts of this ashed material. These additions were all made 6 days per week.

CHART IV. Comparison of hemoglobin-building value of iron from ferric chloride, lettuce ash, and the hydrochloric acid extract of lettuce ash, at two levels of iron intake. These additions were made to the basal diet of cow's whole milk 6 days per week. Rat 511 ♀ was killed at the point indicated because of a peculiar dry, scaly condition affecting the eyes, ears, and paws which we feared might be infectious.

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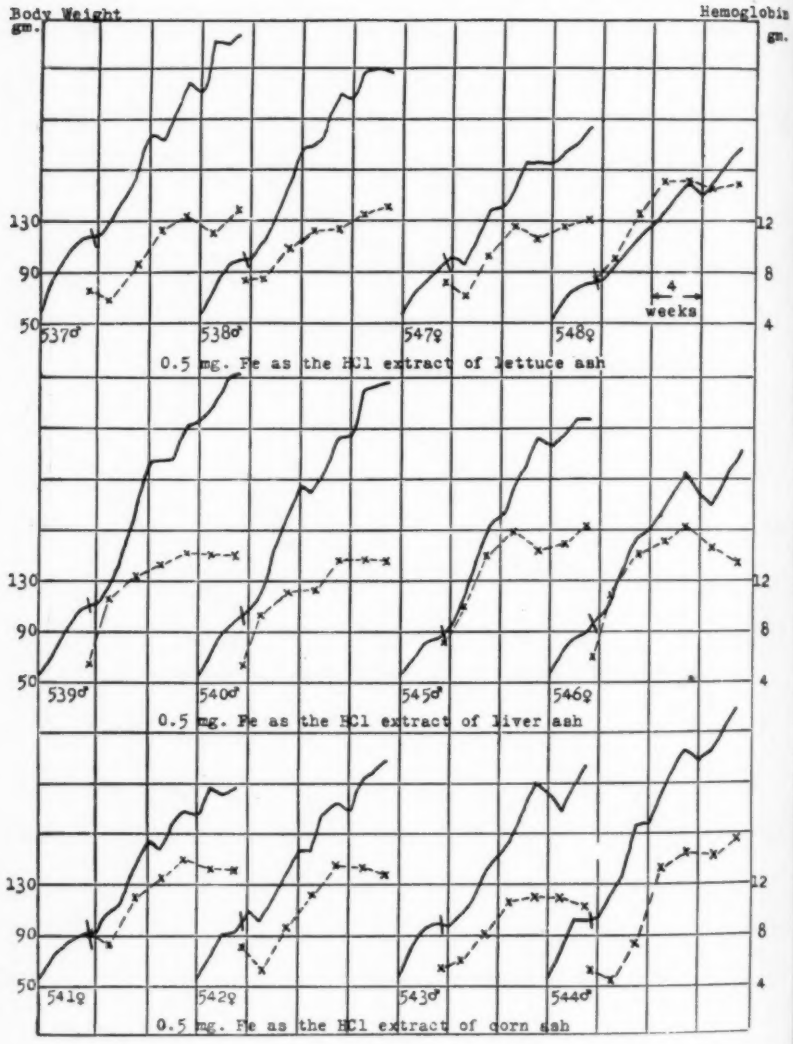

CHART VI. Results of adding to the basal diet of cow's whole milk hydrochloric acid extracts of the ashes of lettuce, beef liver, and yellow corn. These additions were fed to rats at a level such that they introduced 0.5 mg. of Fe 6 days per week.

Attention should be called, however, to those animals which received the HCl extract of the ash of liver ignited with calcium carbonate (Chart V). It will be noted that in all four cases the growth of the animals was markedly depressed for a period of 3 to 4 weeks after the addition was made. Following this period the growth was apparently normal. This was undoubtedly due to the relatively large amount of soluble calcium salts present in this

TABLE I.

Average Hemoglobin Levels, at Time Addition Was Made and at Intervals Thereafter, of Animals Receiving 0.5 Mg. of Fe from Ashes and Ash Extracts of Various Materials Compared with Ferric Chloride.

	Ferric chloride.	Ash of lettuce.	HCl extract ash of lettuce.	Ash of liver + $CaCO_3$.	HCl extract ash of liver + $CaCO_3$.	HCl extract ash of liver.	HCl extract ash of corn.
	Gm. hemoglobin per 100 cc. blood.						
At time of addition.	7.30(8)*	7.36(2)	7.02(6)	5.80(4)	6.19(4)	6.91(8)	6.72(4)
After addition.							
2 wks.	6.10(8)	9.20(2)	7.57(6)	10.31(4)	10.61(4)	10.88(8)	5.86(4)
4 "	6.00(6)	11.01(2)	10.46(6)	13.33(4)	13.21(3)	13.80(8)	9.92(4)
6 "	4.72(5)	12.38(2)	12.56(6)	12.43(4)	11.23(3)	13.19(8)	12.94(4)
8 "	6.32(3)	12.31(2)	13.15(6)	15.46(4)	15.32(3)	14.91(8)	14.30(4)
10 "	5.08(3)	13.13(2)	12.95(6)	15.60(4)	15.62(3)	14.74(8)	13.80(4)

* The figures in parentheses refer to the number of animals used in arriving at the average figure.

particular preparation, a condition sufficient to cause the death of one of the animals, Rat 557. No such untoward effect was noted where an equal quantity of calcium was fed in the form of the ash of liver ignited with calcium carbonate—presumably because of its being present in more insoluble form. In no case, however, in any of these animals which received the calcium-containing preparations is any depression noted in the hemoglobin regeneration.

In comparing the efficiency of the ashes and ash extracts, although all serve to cure the anemic condition in our animals, it appears that the beef liver preparations (Charts IV to VI and Table I) are slightly more prompt in bringing about a cure. The preparations of corn ash and lettuce ash apparently contain the hemoglobin-building substance, or substances, in slightly less concentration.

It appeared to us that the results of all these data which we have presented could best be explained by assuming that, in addition to iron, the ashes and extracts of the ashes of corn, lettuce, and beef liver contained some other substance, or substances, vitally concerned in the building of hemoglobin. What this substance was we did not know but that it was inorganic in nature was definite. Necessarily such a substance would have to be active in exceedingly small amounts.

SUMMARY.

1. Evidence is presented showing that inorganic iron salts (chloride, sulfate, acetate, citrate, and phosphate), when fed at such a level that they introduce 0.5 mg. of Fe daily (six times per week), fail to increase materially the hemoglobin level in anemic rats.

2. The ashed residues from dried beef liver, dried lettuce, and yellow corn, and acid extracts of the same, when fed at such a level that they introduce 0.5 mg. of Fe six times per week, are very effective in curing the anemia produced in rats by a diet of cow's whole milk.

3. These results are explained by assuming that the ashes and ash extracts contain in addition to iron some other inorganic substance, or substances, vitally concerned in the building of hemoglobin.

BIBLIOGRAPHY.

1. Waddell, J., Steenbock, H., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1928, lxxvii, 769.
2. Hart, E. B., Steenbock, H., Elvehjem, C. A., and Waddell, J., *J. Biol. Chem.*, 1925, lxv, 67.
3. Hart, E. B., Elvehjem, C. A., Waddell, J., and Herrin, R. C., *J. Biol. Chem.*, 1927, lxxii, 299.

XUM

4. Mitchell, H. S., and Schmidt, L., *J. Biol. Chem.*, 1926, lxx, 471.
5. Abderhalden, E., *Z. Biol.*, 1900, xxxix, 113.
6. Baudisch, O., and Welo, L. A., *J. Biol. Chem.*, 1925, lxiv, 771.
7. Robscheit-Robbins, F. S., and Whipple, G. H., *Am. J. Physiol.*, 1925, lxxii, 408.
8. Minot, G. R., and Murphy, W. P., *J. Am. Med. Assn.*, 1927, lxxxix, 759.
9. Mitchell, H. S., and Vaughn, M., *J. Biol. Chem.*, 1927, lxxv, 123.

IRON IN NUTRITION.

VII. COPPER AS A SUPPLEMENT TO IRON FOR HEMOGLOBIN BUILDING IN THE RAT.*

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(Received for publication, March 23, 1928.)

In the preceding paper of this series (1) we have presented data showing that iron salts of high purity, when fed at levels of 0.5 mg. of Fe six times per week, were ineffective in correcting a progressive anemia in rats confined to a diet of cow's whole milk; but that an equal amount of iron fed as the ash, or acid extract of the ash, of dried lettuce, of yellow corn, or of beef liver was very potent in restoring to normal the hemoglobin of the blood stream.

When we were in the midst of those investigations, we were fortunate to obtain for experimental purposes—before it appeared on the open market—a generous quantity of a liver preparation[1] from Eli Lilly and Company. This was essentially that fraction of liver, the preparation of which has been described by Cohn and associates (2), which has been found very effective in the treatment of pernicious anemia. We were, naturally, curious to find out whether this would be effective in the treatment of anemia in our animals, particularly in view of the fact that it is low in iron.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

† E. R. Squibb and Sons Fellow.

[1] We want to thank Dr. Cohn, Dr. Murphy, the Harvard University committee, and Dr. Rhodehamel of Eli Lilly and Company for this courtesy.

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Experiments with Lilly Preparation.

We first fed the E. L. preparation[2] to four anemic rats at a level of 0.3 gm. 6 days per week, in two cases supplementing it with

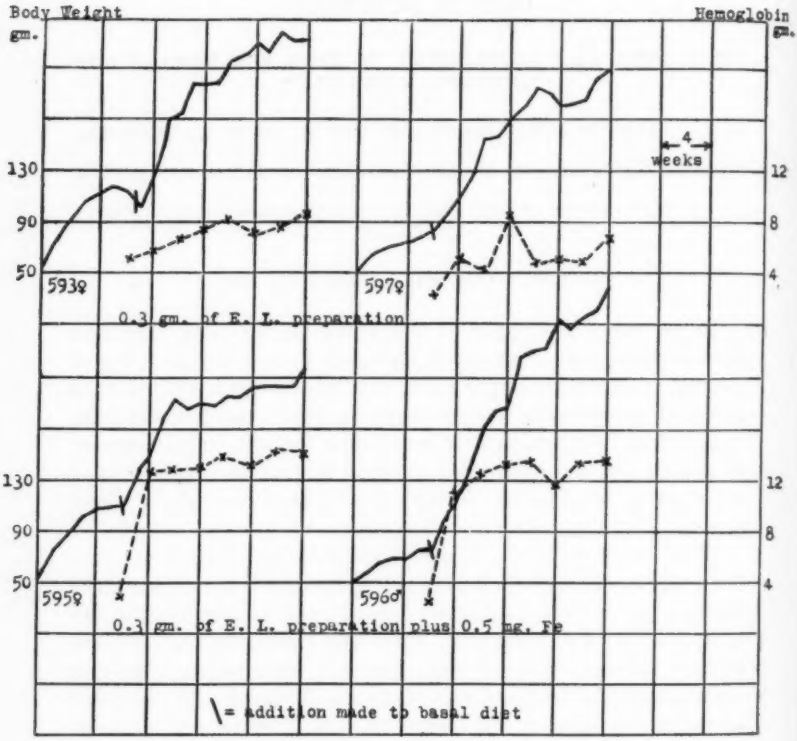

CHART I. In this chart are shown the results secured when there was added to the basal diet of cow's whole milk 0.3 gm. of the Eli Lilly and Company preparation, with and without additional iron. These additions were made 6 days per week. The solid lines in this and the following charts represent body weight, while the broken lines represent hemoglobin in gm. per 100 cc. of blood.

[2] Eli Lilly and Company preparation, Lot No. W 373.

0.5 mg. of Fe as ferric chloride. From the information printed on each vial we considered that 0.3 gm. was approximately equivalent to 1.7 gm. of dried beef liver, which we had used in our previous experiments to introduce 0.5 mg. of Fe. An analysis showed that the E. L. preparation contained only 0.008 per cent Fe. The feeding of 0.3 gm., therefore, introduced only 0.024 mg. of Fe.

The results of this experiment are shown in Chart I. It will be seen that the hemoglobin levels of the animals receiving only the E. L. preparation remained low, while those animals which received iron in addition to the E. L. preparation increased their hemoglobin very rapidly and attained normal levels in a short time. It is interesting, however, to note the marked growth response of the two animals which did not receive extra iron. Outwardly, these two animals appeared almost as thrifty as the two which received the extra iron supplement. We mention this point to emphasize again what we have noted before; namely, that very efficient use is made of small amounts of iron in the diet in the presence of other factors.

Having shown that this E. L. preparation was efficacious in the cure of anemia in our rats we naturally turned our attention to the ash of this material. In the next experiments we fed the ash and a hydrochloric acid digest of the ash to anemic rats. The ash and the acid digest were fed 6 days per week at a level that represented 0.3 gm. of the original E. L. preparation, supplemented in each case by 0.5 mg. of Fe as $FeCl_3$. The quantities for individual feedings were ashed in small crucibles and just prior to being fed, a small amount of milk was added to the ashed residue in the crucible and after standing a few minutes was rubbed up with a stirring rod. The ash was easily dispersed in the milk and consumption was excellent. The hydrochloric acid digest of the ash was prepared by heating the ash in dilute HCl and diluting to definite volume.

In addition to the ash and HCl digest of the ash we also fed a hydrochloric acid extract. The ashed residue from a rather large quantity of the E. L. preparation was first extracted with a small amount of strong HCl and then filtered. The filtrate was evaporated to dryness and then dehydrated in an oven at 110°. This dehydrated mass was in turn extracted with dilute HCl, filtered, and upon dilution to suitable volume was fed to anemic

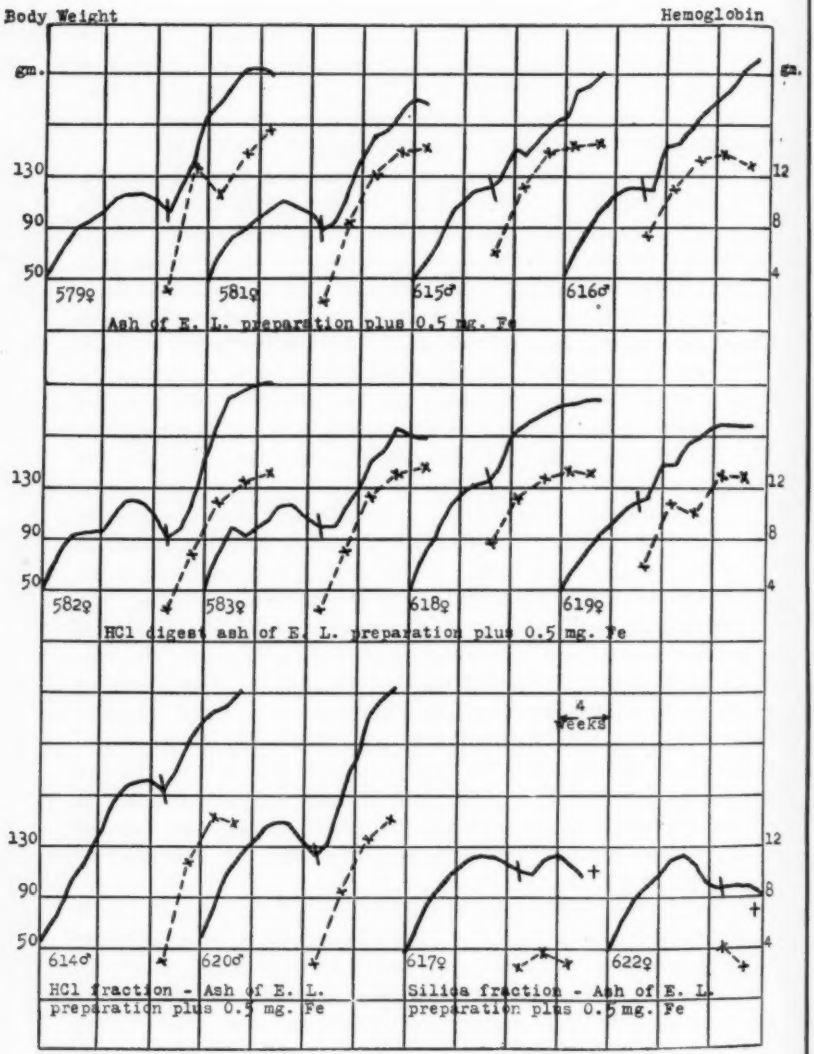

CHART II. In this chart are presented the results secured when there was added to the basal diet the ash, an acid digest of the ash, or fractions of the ash of the E. L. preparation, supplemented in each case with Fe. These additions, each representing 0.3 gm. of the original preparation, were made 6 days per week.

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rats. The residue remaining from this last extraction was suspended in water and likewise fed to anemic rats. It is referred to in Chart II as "silica fraction." Both fractions were fed at a level representing 0.3 gm. of the original E. L. preparation and both were supplemented with 0.5 mg. of Fe as $FeCl_3$.

The results of these experiments on the ash, the HCl digests, and extracts of the ash are presented in Chart II. These data show very conclusively that the ash and HCl digest and extract of the ash were, when supplemented with additional iron, very effective in curing the anemia in our animals. It is also to be noted that the silica fraction was totally inert, supplemented as it was with additional iron. These results suggested that the E. L. preparation might represent a fraction containing in greater concentration that substance (or substances) which we had assumed as being present in the ashes of yellow corn, dried lettuce, and dried beef liver. This seemed particularly true in view of the fact that we had found beef liver to contain the unknown substance in slightly greater concentration than corn or lettuce.

Fractionation of the Ashes.

Quite early in our experiments we had made an attempt to fractionate the acid extract of the ash of lettuce. To accomplish this we treated the ash of a known quantity of lettuce with strong HCl, then filtered and dehydrated it. The dehydrated mass was in turn digested with dilute HCl and the silica filtered off. The filtrate was then treated with ammonia to precipitate the iron hydroxide fraction. The precipitate was filtered off, dried, and fed at a level which introduced 0.5 mg. of Fe. The filtrate from the ammonia precipitation was evaporated to dryness, ignited to get rid of the ammonium chloride, and fed at a level representing an amount of lettuce equivalent to the hydroxide precipitate. The dried residue from the filtrate was, of course, supplemented with 0.5 mg. of Fe as $FeCl_3$. Both preparations were added to the basal diet of anemic rats six times per week.

The results from this fractionation, which are presented in Chart III, show that no clean cut separation of an active from an inactive fraction was made by means of ammonia precipitation. Both fractions were effective in restoring the hemoglobin to normal

levels, although recovery seemed to be more rapid on the ammoniacal precipitate.

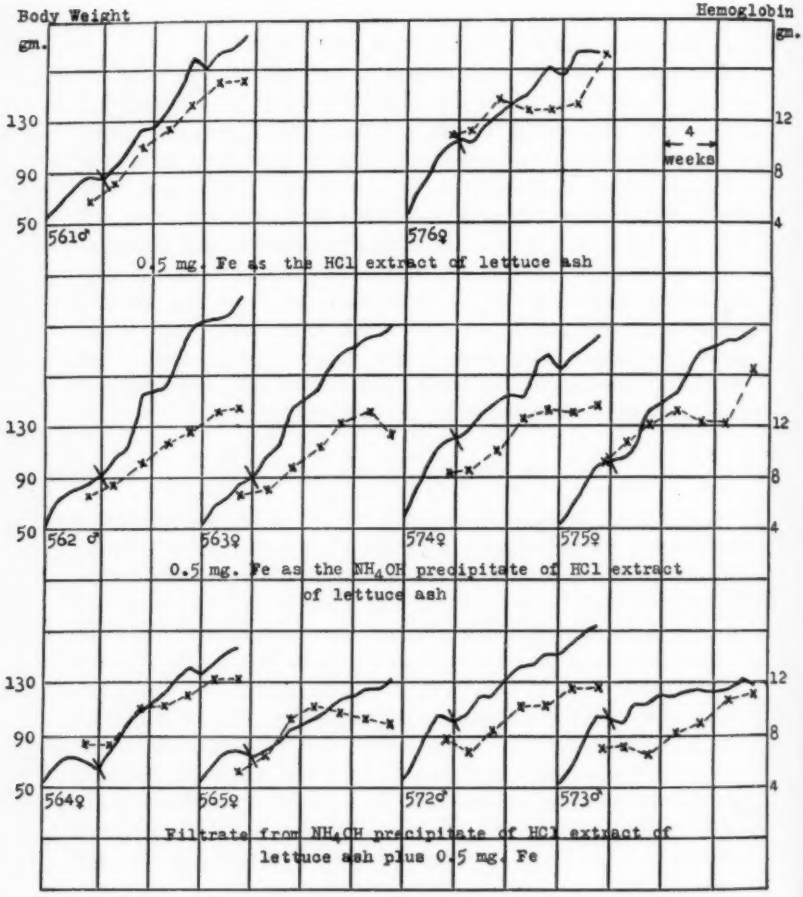

CHART III. In this chart are presented the results of the first attempt to fractionate the hydrochloric acid extract of the ash of lettuce. It will be seen that the material effective in hemoglobin formation is apparently present in both fractions.

With this experience available, in directing our attention to the fractionation of the ash of the E. L. preparation we decided to

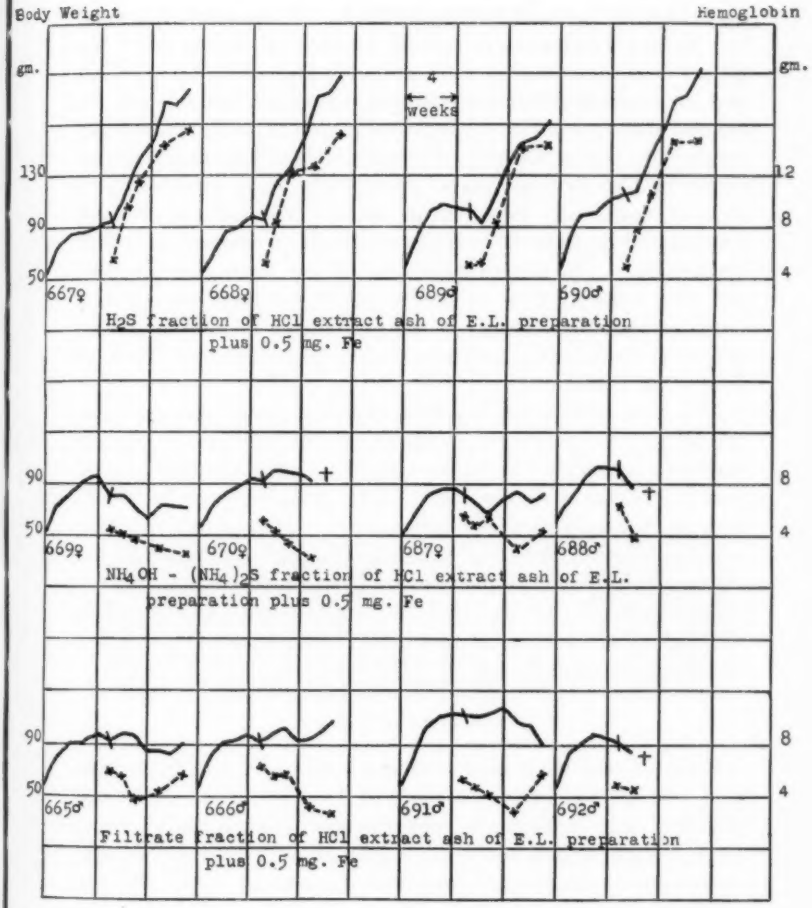

CHART IV. The above are the results secured when there were added to the basal diet of cow's whole milk the three fractions of the hydrochloric acid extract of the ash of the E. L. preparation, supplemented in each case with Fe. These additions were made six times per week.

make it more drastic. To this end we extracted the ash with strong HCl, dehydrated it by heating, digested it with dilute HCl, and filtered off the silica as described and then made our first separation by means of hydrogen sulfide. There resulted a small but distinct precipitate of sulfides which was filtered off. The filtrate was then treated with ammonia and ammonium sulfide and the resultant precipitate also was filtered off. In this way we secured three fractions, (1) H_2S precipitate, (2) NH_4OH-$(NH_4)_2S$ precipitate, and (3) filtrate. The sulfides were dissolved in HNO_3, evaporated to dryness, taken up in HCl, again evaporated to dryness, and finally taken up in water. The NH_4OH-$(NH_4)_2S$ precipitate was dissolved in HCl, evaporated to dryness, and taken up in water. The filtrate was evaporated to dryness and ignited and also suspended in water. All three fractions were fed at levels representing 0.3 gm. of the original E. L. preparation six times per week, being supplemented in each case by 0.5 mg. of Fe as the chloride.

The results secured when these three fractions were added to the basal diet of cow's whole milk of two litters of anemic rats are shown in Chart IV. It will be noted there that the H_2S fraction was the potent one—the others being completely ineffective in causing increased hemoglobin formation. The animals receiving the fraction obtained by precipitation with hydrogen sulfide increased their hemoglobin content in every way comparable to those which had been fed the E. L. preparation or its ash. This, of course, could not be due to a higher iron intake because iron is not precipitated in the effective H_2S fraction.

First Trial of Copper.

Shortly before the time that these fractions of the E. L. preparation were studied a trial was made of the effect of the addition of 0.25 mg. daily of copper as copper sulfate plus 0.5 mg. of Fe as ferric chloride added to our whole milk diet. This was done with an individual rat, No. 621, which had been made anemic and had a hemoglobin content of only 2.68 gm. per 100 cc. of blood. The copper sulfate used was a stock laboratory preparation marked Merck C.P. The $FeCl_3$ was our preparation from standard iron wire.

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We made this trial of a copper salt because it fitted into our scheme of testing all inorganic elements as supplements to iron which have been recognized as present in biological materials. Further, its immediate use was suggested by the fact that we had

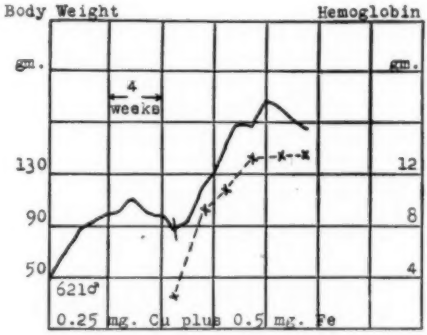


CHART V. The above is the record of Rat 621, which received 0.25 mg. of Cu as copper sulfate and 0.5 mg. of Fe as ferric chloride 6 days per week.

FIG. 1. Rat 621. First attempt in correcting anemia with $FeCl_3$ and $CuSO_4$. This rat was made anemic by a whole milk diet. When the hemoglobin reading was 2.68 gm. per 100 cc. of blood 0.5 mg. of Fe as $FeCl_3$ and 0.25 mg. of Cu as $CuSO_4$ were added to the diet daily. In 2 weeks after these additions the hemoglobin titer was 9.35 gm.; in 4 weeks 10.94 gm.; and in 6 weeks it reached the level of 13.34 gm. Without the copper addition the rise in the hemoglobin would not have occurred.

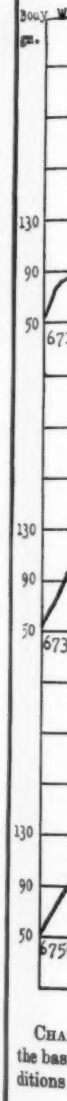
often noticed a pale blue color in the ash of some of the materials studied, particularly the ashes of lettuce and the E. L. preparation. Copper was also suggested by the knowledge that in some of the

molluscs and crustacea it is known to exist as an integral part of the compound hemocyanin, which functions as a respiratory pigment similar to hemoglobin in the higher animals.

The response in this preliminary experiment of copper sulfate feeding was indeed surprising. This preliminary experiment was with but a single animal but the effect was so convincing and helpful that we want to record in Chart V the weight record and hemoglobin curve of this single animal if for no other reason than its historical interest. We think that this is the first experiment (Fig. 1) in the literature giving to copper in association with iron the specific function of hemoglobin building in a mammal on an otherwise satisfactory diet.

After securing these results with the copper-iron-whole milk diet, it becomes possible to discuss intelligently the results secured with the fractionation of the lettuce ash and the ash from the E. L. preparation. As shown in Chart III the fractionation of the ash of lettuce through the use of ammonia gave mixed results; that is, both the ammoniacal precipitate and the filtrate were effective although the precipitate was perhaps somewhat more potent. Copper salts are known to be soluble in ammonia and theoretically should be largely in the filtrate, but when one is dealing with a very considerable colloidal precipitate of $FePO_4$ and small amounts of copper, the chances for adsorption of some of the copper are extremely large; while, on the other hand, some of the copper would go through into the filtrate. In our fractionation with ammonia only one precipitation was carried out, making it altogether probable that the partition of the copper between the $FePO_4$ precipitate and the filtrate is the explanation of the results secured.

We began to surmise that in the fractionation of the E. L. preparation, the silica fraction proved to be *inactive* because of the complete extraction of the copper with HCl; that the H_2S fraction was *active* because of the relatively complete removal of the copper with that reagent and its presence in that fraction; that the NH_4OH-$(NH_4)_2S$ fraction was *inactive* because the copper had been too completely removed with H_2S; and that the filtrate from the NH_4OH-$(NH_4)_2S$ precipitate was *inactive* because the copper had already been removed too completely by the reagents used, particularly the H_2S.



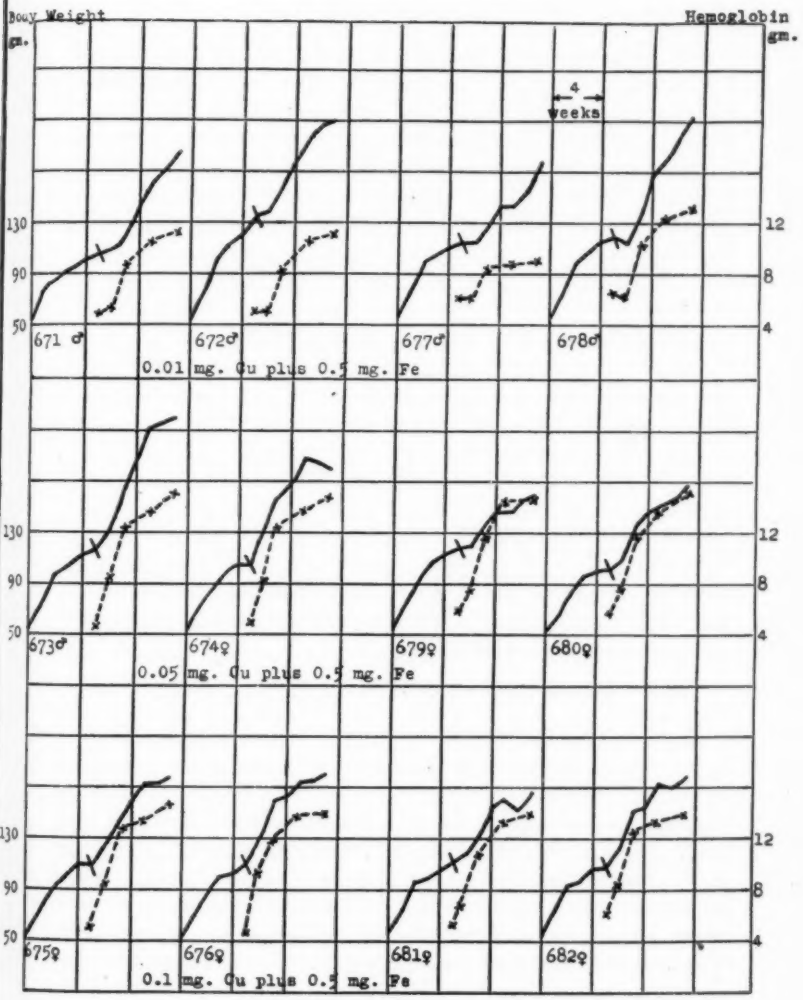


CHART VI. In this chart are presented the data secured when there were added to the basal diet of cow's whole milk three levels of Cu supplemented with Fe. These additions were made six days per week.

Further Experiments with Copper.

After the definite response made by Rat 621 to a whole milk-Fe-Cu diet, other levels of Cu feeding were begun, in one series Merck's C.P. $CuSO_4 \cdot 5H_2O$ being used, and in a second series a specially prepared copper sulfate. This last named $CuSO_4$ was prepared from Kahlbaum's electrolytic sheet copper by dissolving 10 gm. of this in HNO_3, evaporating to a low volume, taking up with warm dilute H_2SO_4, and then letting the solution stand in the ice box to crystallize. The crystals were filtered off, dissolved in H_2O, evaporated to a small volume, and again set aside for crystallization. The feeding experiments were begun with the administration six times per week of 0.01, 0.05, and 0.10 mg. of Cu as the dose per individual rat. 0.5 mg. of Fe as $FeCl_3$ was given in addition. We aimed in this series to work with concentrations of Cu a great deal lower than in our first trial.

The data from these experiments are shown in Chart VI. The animals which received the 0.05 mg. and the 0.1 mg. levels of copper had a very rapid recovery to normal hemoglobin. Those which received only 0.01 mg. of copper soon showed marked increases with a final return to normal. An improvement in physical well being as evidenced by greatly increased appetite, smoother coats, and increased vigor and activity was a pronounced accompanying phenomenon.

Copper Content of the E. L. Preparation.

From the standpoint of the above results, it becomes intelligible how the E. L. preparation might owe its activity to its content of copper. We have already mentioned that the H_2S fraction of the ash was active and that the ash was always of a pale blue color. Actual determination of the copper content of the ash by the xanthate method revealed 0.016 per cent of copper. When we fed our rats 0.3 gm. of the E. L. preparation as such, we gave them an intake of approximately 0.05 mg. of copper daily. This amount when given as copper sulfate, it will be recalled, was corrective when administered as usual with 0.5 mg. of iron daily.

DISCUSSION.

The discovery that copper may be a factor in the building of hemoglobin in a mammal merits some discussion. That copper

occurs in plant and animal tissues has been known for some time, but as far as we know no definite function has been assigned to it except in the case of some molluscs and crustacea where it is a constituent of hemocyanin—a respiratory pigment.

Recently copper has been reported as occurring in blood both by McHargue (3) and by Warburg and Krebs (4)—the latter finding in the serum of human blood an amount approximating 0.0017 mg. of Cu per cc. and an amount of about the same order associated with the serum proteins. They further report Cu as occurring in the blood serum of the dog, cat, rat, guinea pig, frog, chicken, and goose, as well as in association with the serum proteins of these same animals.

From the limited data available in the literature, one would judge that the new-born come into the world fairly well stocked with Cu as well as Fe and during the suckling stage these reserves become exhausted with final dependence for a supply placed on the food material ingested. McHargue points out that the guinea pig is an exception to this rule and probably for the same reason that in this animal there is no prenatal store of iron, the young guinea pig eating vegetable material soon after birth, as it becomes independent of any large supply of milk sooner than most mammals. McHargue gives interesting data on the distribution of Cu in the tissues of cattle, reporting 400 mg. of Cu per kilo of dry matter in the liver of a calf 5 days old and 50 mg. in the same quantity of liver of an ox; while the liver of a calf born dead contained 908 mg. of Cu per kilo of dried material. Of all the organs analyzed, the liver was the richest in Cu. The spleen of the ox contained 16.6 mg. and lean meat but 4 mg. per kilo of dried material.

Recently the use of liver and liver extracts has come into prominence through the work of Minot and Murphy (5) and Cohn and associates (2) in the treatment of pernicious anemia. The Lilly preparation used by us was made essentially according to the directions of Cohn and associates. The fact that this preparation was found effective in the treatment of anemia in the rat exactly as it has been found effective in the treatment of pernicious anemia in man, appeared to us rather significant. Another fact that the effectiveness of this preparation could be duplicated in our experimental animals by the use of copper alone, which is a

constituent of the E. L. preparation, impressed us still more, suggesting more than a casual incidental connection. It is realized that the successful treatment of the two anemias need not necessarily be alike because of the evidence for the excessive hemolytic destruction in some forms, but it is believed that any combination of factors that facilitates the production of hemoglobin when the diet is otherwise entirely satisfactory, cannot help but have an ameliorative, if not corrective, effect in all types of anemia.

How copper exists in plant and animal tissue is unknown, but McHargue (6) was able to show its presence in the ash of a hot 95 per cent alcohol extract of blue-grass. It will be recalled that we showed that the ash of the cold 95 per cent alcohol extract of cabbage or lettuce was effective in correcting anemia when administered together with iron. We have now assayed these extracts for copper and shown it to be present, giving us presumptive evidence that it may have been the copper which was responsible for the effect produced by these preparations. As to the form in which Cu occurs in liver, we have no data, but in the E. L. preparation it presumably is not in alcohol-soluble combination. The form in which it occurs in various materials may have a further *modifying* effect upon the action of Cu.

According to McHargue the Cu content of liver may be very variable, depending upon the age of the animal and possibly upon the food source as well as the species. 300 gm. of fresh ox liver (approximately the daily portion recommended in the Minot-Murphy diet) would contain about 4 mg. of Cu, while 300 gm. of fresh calves' liver would contain according to the limited data of McHargue about 30 mg. of Cu. Six vials of the E. L. preparation in our possession—the daily dosage recommended—would contain about 4 mg. of Cu. Apparently in the method of preparing from liver an active reagent against pernicious anemia as devised by Cohn and his associates, the Cu content of the original liver has become fairly well concentrated in the final product. We are informed that hog liver, which is reported to contain about the same amount of Cu as beef liver, is the main source of this preparation.

We are, of course, in no position to state how much copper a rat needs in order to maintain its synthesis of hemoglobin at a normal

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rate, because milk is well known to contain copper not only as a normal constituent, but frequently as a contaminant from dairy utensils. Supplee and Bellis (7) give the average Cu content of milk as 0.52 mg. per liter with the amount in the milk of individual cows varying from 0.2 to 0.8 mg. of Cu per liter. Assuming a daily consumption of 35 cc. of whole milk for a 50 to 75 gm. rat, the Cu intake according to Supplee and Bellis' data would be in the neighborhood of 0.017 mg. Copper determinations on the milk we have used in these experiments show an amount equal to 0.32 mg. per liter. This determination was made by ashing the milk, precipitating the Cu from an acid solution with H_2S, and finally determining the Cu by the xanthate method. With a 35 cc. daily consumption the amount of Cu ingested would have been approximately 0.010 mg. By supplementing this milk with a daily administration of 0.3 gm. of E. L. preparation with its Cu content of 0.05 mg., the total Cu intake in the corrective diet would be about 0.06 mg.

How copper in the presence of iron and an otherwise satisfactory diet functions in hemoglobin building is almost idle speculation. So far as the literature shows, hemoglobin does not contain copper. We might advance the orthodox suggestion that the copper acts as a catalyzer for some reactions concerned in hemoglobin building just as iron functions in the production of chlorophyll, although it is not a constituent of the chlorophyll molecule. We believe that our work emphasizes the necessity for the intensive study of the action of small amounts of inorganic elements in nutrition. Only when some important function is involved in a type of reaction that lends itself to ready observation or quantitative measurement are the conditions suitable for making progress in this attractive field.

SUMMARY.

An Eli Lilly and Company preparation from liver, manufactured under the auspices of the Harvard committee for the treatment of pernicious anemia in man, was found effective in the cure of anemia in rats kept on a whole milk diet supplemented with iron.

The ash obtained by the incineration of the Lilly preparation was also active.

HCl extracts of the ash of lettuce, previously reported active, could not be separated sharply into an active and inactive fraction by precipitation with ammonia.

HCl extracts of the ash of a Lilly preparation treated with H_2S, followed by NH_4OH-$(NH_4)_2S$, concentrated the potency in the H_2S fraction.

Copper, as one of the elements to be found in the H_2S fraction of the Lilly preparation, showed itself to be highly active.

We have secured no evidence for the existence of an organic factor necessary for hemoglobin synthesis in rats kept on a whole milk diet supplemented with 0.5 mg. of iron daily.

Our experiments point to the need for a more intensive study of the rôle of small amounts of inorganic substances in the diet.

BIBLIOGRAPHY.

1. Waddell, J., Elvehjem, C. A., Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, 1928, lxxvii, 777.
2. Cohn, E. J., Minot, G. R., Fulton, J. F., Ulrichs, H. F., Sargent, F. C., Weare, J. H., and Murphy, W. P., *J. Biol. Chem.*, 1927, lxxiv, p. lxix; *Proc. Am. Soc. Biol. Chem.*, 1927, vii, 69.
3. McHargue, J. S., *Am. J. Physiol.*, 1925, lxxii, 583.
4. Warburg, O., and Krebs, H. A., *Biochem. Z.*, 1927, cxc, 143.
5. Minot, G. R., and Murphy, W. P., *J. Am. Med. Assn.*, 1926, lxxxvii, 470.
6. McHargue, J. S., *Ind. and Eng. Chem.*, 1927, xix, 274.
7. Supplee, G. C., and Bellis, B., *J. Dairy Sc.*, 1922, v, 455.

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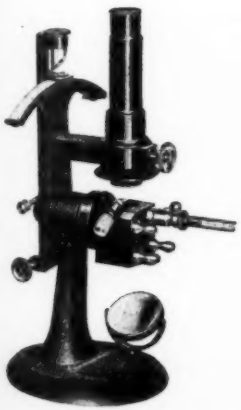

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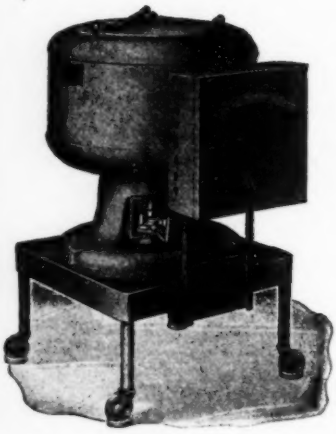

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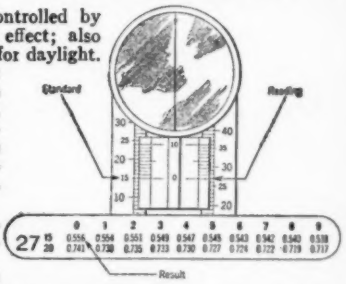

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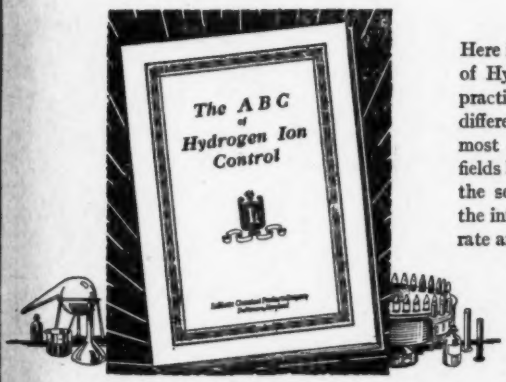

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